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Dispatches from the DMZ: Bottle Cell Formation During Xenopus Gastrulation

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Abstract

Bottle cell-driven blastopore lip formation externally marks the initiation of gastrulation in amphibian embryos. The blastopore groove is formed when bottle cells undergo apical constriction and transform from cuboidal to flask-shaped. Apical constriction is sufficient to cause invagination and is a highly conserved mechanism for sheet bending and folding during morphogenesis; therefore, studying apical constriction in *Xenopus* bottle cells could provide valuable insight into this fundamental shape change. Initially described over a century ago, the dramatic shape change that occurs in bottle cells has long captured the imaginations of embryologists. However, only recently have investigators begun to examine the cellular and molecular mechanisms underlying bottle cell apical constriction. Bottle cell apical constriction is driven by actomyosin contractility as well as by endocytosis of the apical membrane. The Nodal signaling pathway, Wnt5a, and Lgl1 are all required for bottle cell formation, but how they induce subcellular changes resulting in apical constriction remains to be elucidated. *Xenopus* bottle cells now represent an excellent vertebrate system for the dissection of how molecular inputs can drive cellular outputs, specifically the cell shape change of apical constriction.

Introduction

At the beginning of amphibian gastrulation, a thin, dark crescent forms at the dorsal marginal zone (DMZ), extending laterally and ventrally to form the blastopore (Figure 1). The cells that comprise the blastopore lip are the bottle cells, which are the first cells to undergo evident shape changes during gastrulation. Bottle cells form at the border of the involuting marginal zone and the vegetal cells, over the span of six to eight tiers of cells[1]. Despite their continuous appearance, the first bottle cells are not necessarily neighbors nor are they contiguous[1]. As they apically constrict, bottle cells undergo a dramatic shape change from cuboidal to flask-shaped (Figure 1), hence their name. The constriction event also concentrates pigment granules at bottle cell apices, providing a natural marker for easy identification. Developmentally, *Xenopus laevis* bottle cells are endodermal and contribute to the archenteron wall during gastrulation, eventually lining the liver in tailbud stage embryos[1]. In other amphibians, bottle cells appear to play a more central role. For example, axolotl bottle cells are mesodermal and contribute to head mesenchyme[2].

Xenopus bottle cells undergo apical constriction, an important, widely conserved cell shape change that is central to epithelial sheet bending and invagination[3]. During apical constriction, the apical surface of a cell actively shrinks; when a group of cells does this in a

concerted fashion, a groove or invagination forms[4, 5]. In the case of *Xenopus* bottle cells, apical constriction functions to form the blastopore. Due to their accessibility and quantifiable shape changes, bottle cells are an excellent vertebrate model for studying apical constriction.

Apical constriction is central to gastrulation, neurulation, and organogenesis and may also underlie cell shape changes associated with metastatic cancer[6]. The term "bottle cell" is formally used to describe apically constricting cells in amphibian, sea urchin (primary mesenchyme cells)[7], and white sturgeon[8] embryos, but the process of apical constriction is widely employed by embryos to achieve cell ingression[9], tissue invagination[10], and neurulation[11]. Therefore, it is a matter of embryological and historical context whether an apically constricting cell is called a "bottle cell." As comprehensive reviews on apical constriction in other developmental systems have recently been published[3, 12], only apical constriction in the context of bottle cells will be discussed here.

Bottle Cell Embryology

Early Studies

First described by Rhumbler in 1899[13], bottle cells were also referred to as flask, club, radish, and wedge cells. Ruffini noted that wedge-shaped cells were found in a variety of invaginating tissues, such as the neural tube, otic and optic placodes, and presumptive mouth[14]. Therefore, he hypothesized that the shape change associated with bottle cells was a major driving force during tissue folding.

Due to their dramatic shape change, bottle cells were assumed to play a major role during amphibian gastrulation. This notion was supported by the findings of Holtfreter, who performed the first detailed description and analysis on morphogenesis in amphibians. Working mainly with the salamander *Amblystoma punctatum* (now known as *Ambystoma maculatum*), Holtfreter noted that isolated blastoporal cells in alkaline conditions made lamellipodial extensions basolaterally, reminiscent of migratory cells in culture[15]. This was consistent with the dominant view at the time that the higher pH in the blastocoel provided a chemotactic cue to direct gastrulation movements. In addition, Holtfreter observed that isolated blastoporal cells could invade into an endodermal substratum and cause an invagination[15] (Figure 2), whereas other types of cells invaded without driving invagination. He concluded that blastoporal cells (i.e., the bottle cells) were capable of causing invagination, and that after the initial blastopore formation, bottle cells then became migratory in nature to drive involution by dragging the neighboring cells into the embryo.

The Contribution of Bottle Cells Redefined

For decades, Holtfreter's model of bottle cells as the central driving force during amphibian gastrulation remained unchallenged. In 1975, Cooke performed a straightforward experiment to test the function of bottle cells: he removed bottle cells from *Xenopus laevis* embryos to determine their contribution during gastrulation. Surprisingly, the majority of embryos gastrulated and developed normally, with only a few showing head defects[16]. As

intriguing as Cooke's findings were, the results were not well documented, i.e., no drawings of the embryos were published, nor were the surviving embryos analyzed.

In a seminal study, Keller definitively showed that bottle cells contribute only to the initial blastopore invagination in *Xenopus* and do not play a role in other gastrulation movements[17]. Following removal of bottle cells, scanning electron micrographs (SEM) showed that a dorsal groove still formed. The resulting embryos exhibited a shorter, wider archenteron, suggesting a role for bottle cells in archenteron length. Even though bottle cells do not play the central role proposed by Holtfreter, Keller suggested that they might be important for gastrulation efficiency by shrinking the blastopore surface area by 10-fold.

To determine what shape changes were intrinsic to bottle cells, Hardin and Keller cultured dorsal lip explants and performed SEM to analyze cell shapes. Interestingly, explanted bottle cells still apically constricted but did not undergo apicobasal elongation[1]. Additionally, a cup-shaped pit formed instead of a deeper groove[1], indicating that while apical constriction is capable of bending the tissue, additional morphogenetic processes (e.g., vegetal rotation[18] and marginal zone involution[19]) provide forces that shape the invagination into the blastopore groove. The depth of the pit formed by explanted bottle cells was indistinguishable from the blastopore groove created by intact bottle cells[20], suggesting that apical constriction is the major factor driving blastopore invagination. Explanted bottle cells also appear to undergo their normal behavior of respreading following constriction[1]. It is unknown how the bottle cells respread, but the authors hypothesized that if bottle cells were to display any migratory behavior, it would be during this respreading phase. In conclusion, apical constriction is an intrinsic cell behavior of bottle cells and their constriction initiates a groove that may contribute to hoop-stress, facilitating more efficient involution of the marginal zone.

The Cellular Basis of Shape Change in Bottle Cells

As discussed above, apical constriction in bottle cells is an intrinsic behavior, while apicobasal elongation is not[1]. In other systems, apical constriction is driven primarily by actomyosin contractility[3]. Transmission electron microscopy (TEM) of amphibian bottle cells supported an actomyosin contractility-based model, as the micrographs showed dense fibrous structures at the subapical region that resembled actin microfilaments[21, 22]. Phalloidin staining of bottle cells confirmed that F-actin does indeed accumulate at the apical membrane in constricting bottle cells[23]. Additionally, TEM studies showed that the bottle cell apical membrane folds into microvillar structures[21, 22], suggestive of an active contraction of the apical surface.

The actomyosin contractility model was not tested rigorously in *Xenopus* bottle cells until recently. This was perhaps due to the perception that the yolky cells of *Xenopus* are challenging to image. We set out to establish methods for improved imaging in order to determine the cytoskeletal dynamics required for bottle cell apical constriction. We showed that F-actin and myosin are enriched apically in bottle cells (Figure 3), and that perturbing F-actin and myosin activity using pharmacological inhibitors prevented apical

constriction[20]. In addition, we found that microtubules were localized in apicobasal arrays (Figure 3), as was seen in earlier TEM studies[22].

Surprisingly, microtubules were not involved in apicobasal elongation as was hypothesized; instead, they were required for apical constriction. What role, then, do microtubules play during apical constriction? In our microtubule inhibitor studies, we found that stabilizing microtubules with taxol did not affect apical constriction, whereas depolymerizing microtubules using nocodazole specifically inhibited apical constriction. Since both drugs affect microtubule dynamics, our findings showed that intact but not dynamic microtubules were required for apical constriction, suggesting a structural role for microtubules. As microtubules are required for intracellular vesicle trafficking, and because TEM studies showed an abundance of small vesicles at the apical region of bottle cells[21, 23], we hypothesized that microtubules could act to facilitate endocytosis of the shrinking membrane during apical constriction. We confirmed that endocytosis occurs in the bottle cells and that bottle cells are the only cells in the early gastrula undergoing endocytosis of apical membrane[24]. Furthermore, through perturbing the activity of the endocytic GTPase Dynamin, we found that endocytosis was required for apical constriction, specifically during the latter part of constriction to remove excess membrane[24]. These studies were the first to identify endocytosis as a contributor to cell shape changes and highlighted the importance of membrane remodeling during morphogenesis. A schematic summarizing the cellular mechanisms driving bottle cell apical constriction is shown in Figure 4. Interestingly, the inhibition of Dynamin2 in MDCK cells causes cells to apically constrict[25]. The mechanism appears to be independent of endocytosis; instead, Dynamin2 interacts with Cortactin to facilitate actomyosin dynamics at the apical membrane[25]. This is clearly a very different mechanism from what we found in *Xenopus* bottle cells, thereby demonstrating the diverse strategies used by cells to accomplish various tasks.

Despite the dramatic apicobasal elongation seen in bottle cells and other apically constricting cells, there is strong evidence that cell lengthening is not an intrinsic behavior in bottle cells. As discussed above, elongation does not occur in explanted bottle cells[1], suggesting that bottle cell lengthening is likely a passive byproduct of forces from neighboring tissues. Furthermore, apical constriction can be disrupted without any effect on elongation[20], indicating that the two processes are not causally related in bottle cells. More generally, it is unknown what, if any, contribution cell elongation makes toward tissue invagination. In the neural tube, elongation during constriction appears to be an active process that is independently controlled[26]; therefore, elongation may play a more central role in some cases of invagination and sheet bending than in others.

Molecular Mechanisms of Apical Constriction in Bottle Cells

To date, Shroom3[27] and Nectin[28] are the only genes known to be necessary and sufficient for apical constriction in vertebrates; however, at least in the case of Shroom3, the gene is not expressed in bottle cells and a dominant interfering construct has no effect on bottle cell apical constriction[27]. Therefore, other gene(s) must be responsible for activating bottle cell apical constriction. The TGF-β superfamily of ligands, and in particular Nodal, is a strong, upstream candidate for regulating bottle cell shape changes. Overexpression of the TGF-β

superfamily ligands Xnr1, Activin, and BVg1, upstream activator VegT, as well as the receptor-Smad Smad2 are all capable of inducing ectopic bottle cells[23, 29, 30]. These ectopic bottle cells strongly resemble endogenous bottle cells in their temporal and ultrastructural similarities: they form at the same time, apically accumulate pigment granules and F-actin, and contain microvillar structures at the apical membrane[23]. Just as activating Nodal signaling can induce ectopic apical constriction, inhibition of Nodal can block endogenous bottle cell formation. Overexpression of Cerberus, an inhibitor of Nodal, BMP4, and Xwnt8, blocks blastopore formation, as does overexpression of a dominant negative (dn) Smad2[23, 30, 31]. Moreover, overexpression of Cerberus-short, a truncated version of the protein that specifically inhibits Nodal, can block both endogenous bottle cell formation as well as Xnr1 (Xenopus Nodal-related 1), Xnr2, and Xnr4 induction of ectopic bottle cells[32]. Finally, Xnr1,2 is expressed in the cells adjacent (animally) to the bottle cells, initially in the dorsal organizer, progressing laterally and ventrally as the blastopore forms[33]. Xnr1,2 double morphants display significant gastrulation defects, including delayed/incomplete blastopore closure and failure of convergence and extension[34]. Whether or not bottle cells constrict normally in Xnr1,2 double morphants was not reported, so it will be of interest to determine if Xnr1,2 are necessary for bottle cell formation. Shroom3 facilitates apical constriction through the recruitment of actin, myosin, and ROCK to the adherens junction; therefore, it will be informative to determine whether Xnr1,2 work to recruite a Shroom3-like molecule or in a completely distinct mechanism.

Originally identified in *Drosophila* as a neoplastic tumor suppressor[35], Lgl (*lethal(2) giant larvae*) has since been implicated in a wide variety of cell biological processes such as cell polarity[36] and vesicular trafficking[37]. In a study on *Xenopus* epithelial polarity, Lgl1 was unexpectedly identified as an activator of bottle cell apical constriction[38]. Overexpression of Lgl1 induces ectopic bottle cells[38], whereas knocking down Lgl1 with a morpholino oligonucleotide (MO) inhibits endogenous bottle cell formation[39]. Similarly, Wnt5a overexpression also causes ectopic apical constriction, whereas dnWnt5a suppresses bottle cell formation[39]. Lgl1 appears to act downstream of Wnt5a because Lgl1 overexpression can rescue dnWnt5a-inhibition of bottle cells. Furthermore, either dnWnt5a or Lgl1 MO suppresses Activin-induced ectopic bottle cells[39], suggesting that both Wnt5a and Lgl1 function downstream of TGF-β signaling. Interestingly, Lgl1 protein levels appear to be regulated by Dishevelled through Frizzled8[38], implicating multiple Wnt pathway components in this process. Moreover, as *frizzled* was shown to be important for activating actomyosin contractility during *C. elegans* apical constriction[40], it is possible that Wnt signaling may play a conserved role during apical constriction.

Conclusions

Amphibian bottle cells, with their dramatic cell shape changes, have fascinated embryologists for over a century. Although they do not play a pivotal role during gastrulation as hypothesized by Holtfreter, *Xenopus* bottle cells are nonetheless an excellent model for studying apical constriction in a vertebrate system. The recent investigations into the cellular and molecular mechanisms driving bottle cell formation implicate some well-known processes (e.g., actomyosin contractility[3, 12], Wnt signaling[40]) as well as a few less-studied mechanisms (e.g., endocytosis, Nodal, Lgl1).

Although the basic features of bottle cells have been described, much remains to be investigated. In other apically constricting cells, adherens junctions are crucial for maintaining tension as the actomyosin machinery contracts[41]. Therefore, it is very likely that adhesion plays a major role during bottle cell apical constriction and should be a focus for future research. Additionally, it is unknown whether the actomyosin contractility manifests itself as a meshwork or a purse-string, which are the two major mechanisms that have been described to drive apical constriction[12]. *Drosophila* ventral furrow formation is a primary example of meshwork-driven apical constriction. Here, myosin is recruited to the apical F-actin mesh and its localized activation causes contraction or shrinking of the mesh[12]. Recently, it was shown that the actinomyosin machinery does not contract continuously, but does so in pulses, like a ratchet[42]. It will be of interest to determine if this ratchet mechanism is also at work in *Xenopus* bottle cells. In contrast, a purse-string mechanism causes apical constriction in the *Xenopus* neural tube, in which actin, myosin, and ROCK are recruited to the adherens junctions by Shroom3[43, 44], creating a circumferential belt. When this belt undergoes actomyosin contractility, it “cinches” up the membrane, much like a purse-string, and shrinks the apical membrane. Finally, in addition their structural function during endocytosis, microtubules could play important roles during apical constriction. In *Drosophila* S2 cells, the microtubule plus-end binding protein EB1 directs DRhoGEF2 toward the cortical membrane to cause actomyosin contraction[45]. It is possible that microtubules could also actively transport molecules important for *Xenopus* bottle cell apical constriction. Knowing how the cytoskeleton interacts with adherens junctions, the cortical membrane, and small GTPases will be key to expanding our understanding of the cellular mechanisms underlying apical constriction.

Molecularly, it will be important to identify the genes that regulate apical constriction in bottle cells. Since Nodal signaling is required for bottle cell formation, a potential approach is to screen downstream transcriptional targets to identify genes required for bottle cell formation. Another important question to address is how Lgl1, a basolaterally localized protein, functions during apical constriction. Is Lgl1 directly interacting with the cytoskeleton, or is it functioning to establish apical-basal membrane domains to indirectly facilitate apical constriction?

Some of the most fundamental questions about bottle cell apical constriction remain unanswered. For example, how are bottle cells initially specified? What factors regulate the precise timing of constriction around the marginal zone, and is this timing even important? Local heating of the ventral marginal zone caused bottle cells to form ventrally first and then dorsally, with no effect on gastrulation or neurulation[46], suggesting that timing of bottle cell formation is not linked with later cell fates or behaviors. As apical constriction is an inherently polarized cell behavior, what is the role of apical-basal polarity during apical constriction? How do bottle cell-specifying genes ultimately cause reorganization and activation of the cytoskeleton? Now that we have some clues into the molecular and cellular mechanisms responsible for bottle cell apical constriction and the experimental tools to study this process, we can begin to address these fundamental questions.

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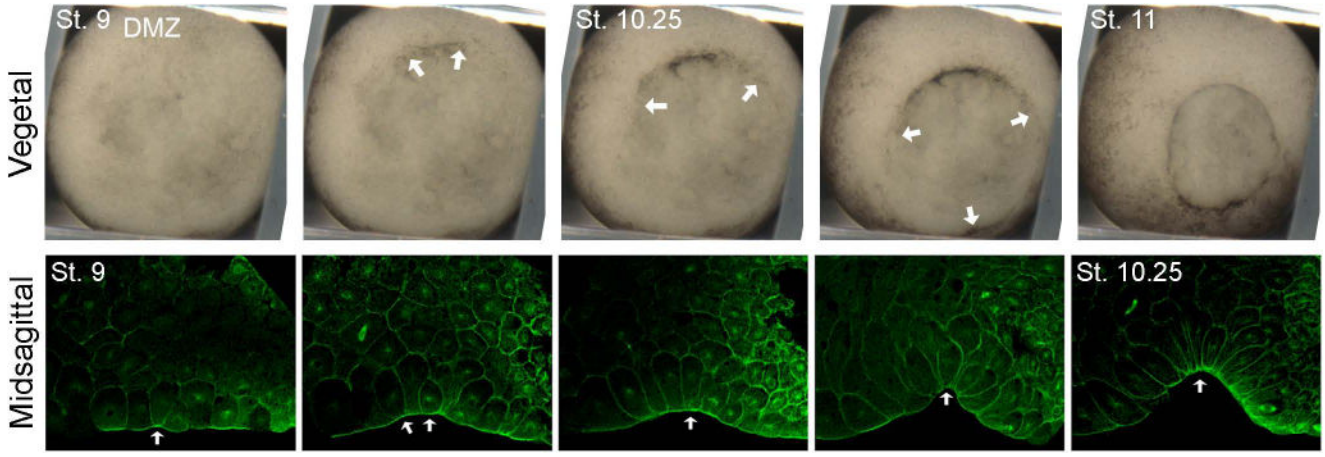

Figure 1.

Bottle cell formation as the first external sign of *Xenopus* gastrulation. Top, vegetal view of blastopore formation, with bottle cells forming initially in the dorsal marginal zone (DMZ), then laterally and ventrally to form the circular blastopore. Arrows mark the extent of apically constricting bottle cells. Bottom, midsagittal confocal images of bottle cells immunostained with α-tubulin antibody. Embryos are oriented apical down and animal to the right. Arrows point to center of blastopore invagination. St., stage. (Reprinted from Lee and Harland, 2007[20]).

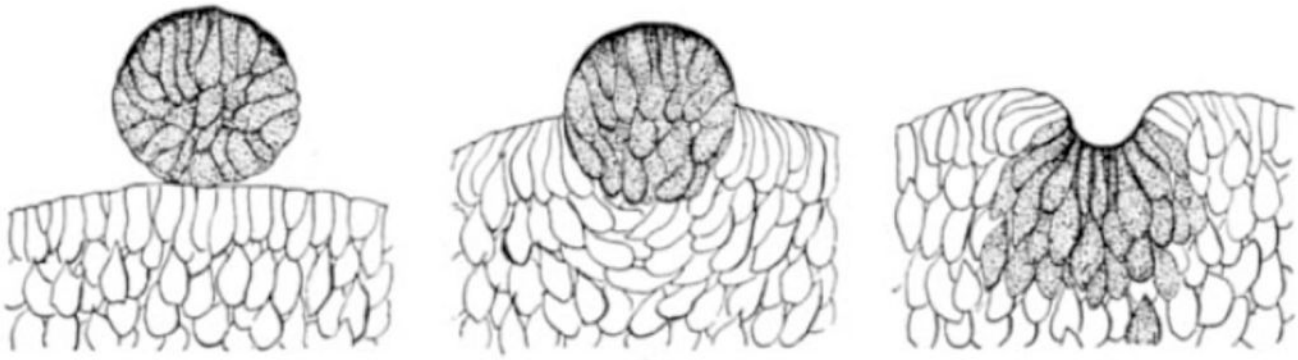

Figure 2.
Holtfreter's classic experiment of blastoporal cells incorporating, then invaginating, into an endodermal substrate. (Reprinted with permission from A Study of the Mechanics of Gastrulation, Part II, by Johannes Holtfreter, JD Wiley and Sons, 1944).

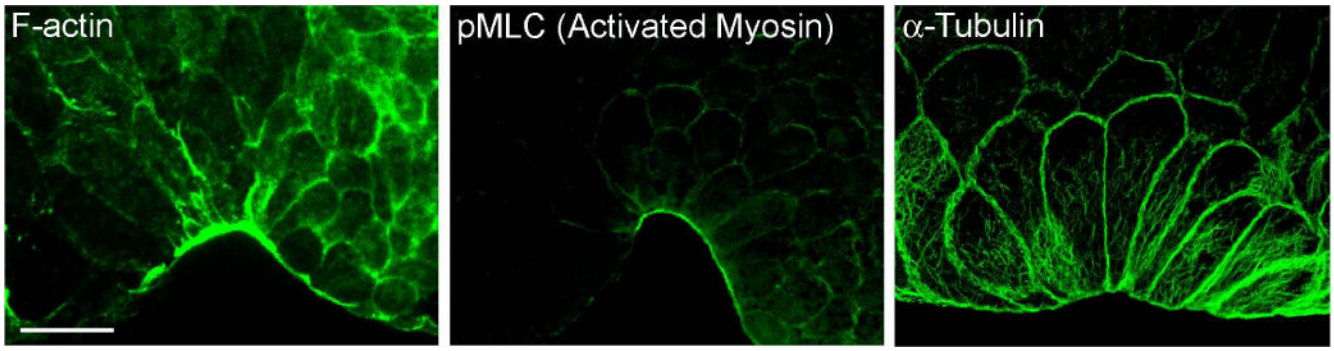

Figure 3.

F-actin and activated Myosin (pMLC) are enriched apically in bottle cells, whereas microtubules (α-tubulin) are arranged in apicobasal arrays. Midsagittal confocal images; embryos are oriented apical down and animal to the right. Scale bar = 50μm. (Reprinted from Lee and Harland, 2007[20]).

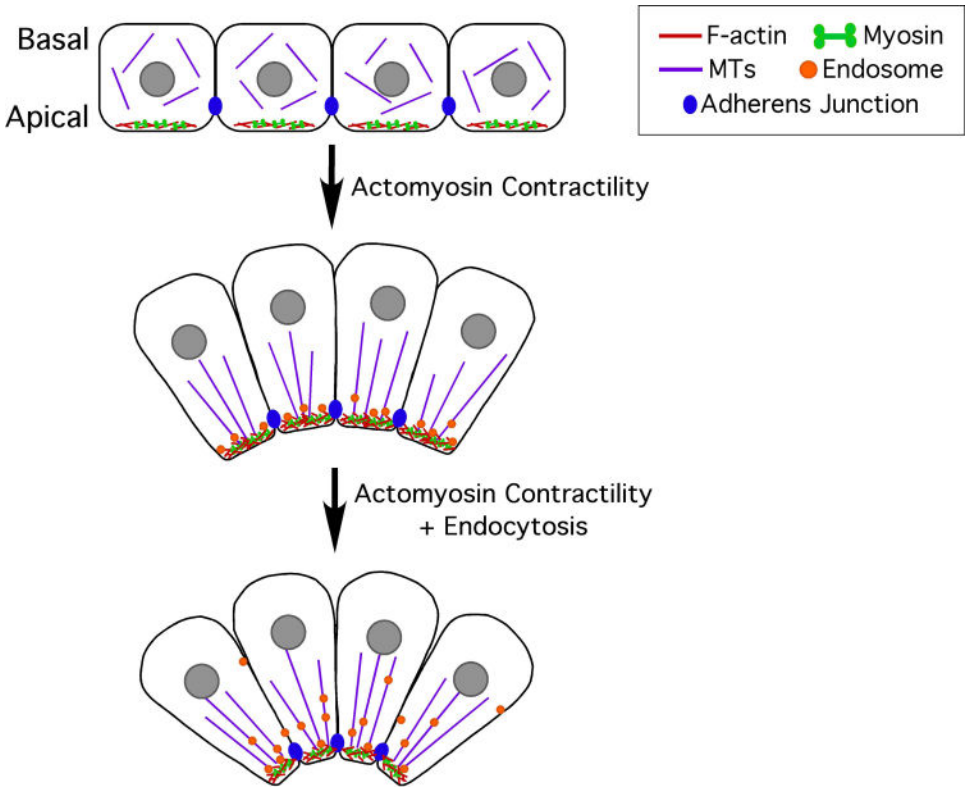

Figure 4.

Schematic of the cytoskeletal mechanisms underlying bottle cell apical constriction, as described[20, 24]. Actomyosin contractility is the main driving force, but endocytosis is also required for efficient constriction later in the process. Adherens junctions have not been directly implicated, but are drawn in at their presumed subcellular location.