Arp2/3 complex function in the epidermis

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Abbreviations: Arp, Actin-related protein; FRAP, fluorescence recovery after photobleaching; AJ, adherens junction; N-WASP, neural Wiskott-Aldrich syndrome protein; ZO, zonula occludens; FAK, focal adhesion kinase; EGFR, epidermal growth factor receptor

Introduction

The F-actin cytoskeleton provides scaffolding for cell shape and organization and its dynamics drive membrane and vesicle movement. While studied in much detail in cultured cells, we still have little understanding of how cells within tissues organize their F-actin networks and the specific functions that these provide, especially in differentiated cells. The Arp2/3 complex is a nucleator of branched F-actin filaments.1,2 Originally identified biochemically as a profilin-binding protein,3 this 7-polypeptide complex contains 2 proteins that are structurally similar to G-actin, as well as 5 additional subunits. It was soon hypothesized to promote F-actin assembly by forming a seed to overcome the kinetic barrier of nucleation.4 The first evidence for this came from its subsequent purification as a factor that induced F-actin assembly on Listeria monocytogenes, a bacterium that moves around in the host cytoplasm through localized actin polymerization.5 Subsequent work demonstrated that the Arp2/3 complex was activated by the Listeria protein, ActA,6 which set off a period of very successful identification of cellular proteins that promoted Arp2/3 activity, now called nucleation promoting factors.7-10 Since that time, a great deal of work has elucidated the structure of the Arp2/3 complex, its mechanisms of regulation, and its functions in cultured cells, as reviewed in.2,11 However, there remains a very significant gap in our understanding of the functions of the Arp2/3 complex in intact tissues during development, homeostasis and disease.

In contrast to its essential role in yeast,12 recent studies in multicellular organisms have demonstrated that the Arp2/3 complex is not required for cell viability in a number of in vivo contexts. Rather, loss of Arp2/3 components results in more subtle and often unexpected phenotypes. These range from defects in gastrulation in C. elegans and cell fate determination in Drosophila to alterations in synaptic plasticity in mouse.13-15 Below I discuss major roles of the Arp2/3 complex that have been proposed from studies in cultured epithelial cells. We also discuss emerging data from genetic studies in living animals, with a focus on our recent analysis of loss of Arp2/3 complex activity in the epidermis.16

Of note when reading this commentary is that Arp2/3 complex inhibition has been experimentally induced using a number of methods – small molecule drugs, dominant-negative versions of nucleation promoting factors, and knockdown or genetic disruption of core Arp2/3 complex components. These diverse methods each have their strengths and weaknesses and makes some comparative analysis of the literature difficult. In our analysis of the epidermis, we used mice in which the ArpC3 subunit of the Arp2/3 complex was lost.13,16 Previous work suggests that this results in a complex with about a 12-fold reduction in actin nucleation activity.17 Therefore, it can be seen as a very strong hypomorph and is expected to give results similar to strong knockdown or drug inhibition, but the remaining complex is intact. ArpC3 loss also resulted in mislocalization of other Arp2/3 components.16 It will be interesting in the future to determine whether loss of other subunits that cause complete loss of activity results in distinct phenotypes.

Arp2/3 Complex and Cell-Cell Adhesions

Adherens junctions

Adherens junctions are dynamic cell-cell adhesions that interact with the underlying F-actin cytoskeleton. A number of
studies in cultured simple epithelial cells have documented colocalization of Arp2/3 complex with adherens junctions and physical interactions of Arp2/3 with adherens junctions proteins such as E-cadherin. In addition, both in vitro and cell culture experiments have demonstrated that Arp2/3 complex is responsible for at least some of the F-actin assembly around adherens junctions. Much of this work focused on the initial assembly of adherens junctions where Arp2/3-mediated actin assembly is likely to promote cell-cell contacts and adhesive formation. In contrast, the evidence on functional roles for the Arp2/3 complex in the adhesive strength of mature junctions is more complicated. When the Arp2/3 complex was inhibited in CHO cells by expression of a dominant-negative construct of the nucleation promoting factor N-WASP, there were no defects in adhesive strength noted. In addition, knockdown of Arp3 in a transformed epidermal cell line did not cause defects in cortical localization of adherens junctions. However, knockdown of Arp3 in Caco-2 cells (intestinal epithelial) resulted in decreased tension on cell contacts as measured by rates of recoil after laser ablation. In contrast, inhibition of Arp2/3 complex activity in cultured endothelial cells (which have VE-cadherin based junctions) caused apparent adhesion defects. Therefore Arp2/3 complex function at adherens junctions may vary in a cell-type specific manner.

Surprisingly, in the skin, we found no evidence for adherens junction defects upon loss of ArpC3. There was no change in steady state cortical levels of adherens junction proteins in cells or tissue, no delay in the assembly of new adherens junctions in calcium shift assays, and no change in the turnover of α-catenin at cell junctions by FRAP analysis. This demonstrates that ArpC3 is not required for robust AJ function and is consistent with earlier studies that suggested formins may be the relevant actin nucleator at keratinocyte adherens junctions. While there may be cell-type specificity in the actin nucleator used, another intriguing possibility is that different types of cadherin-based structures use different actin nucleators. While most simple epithelial cells have both zonula adherens junctions at their apical surface and spot adherens junctions along their lateral domains, basal keratinocytes lack a zonula adherens. It is therefore possible that Arp2/3 is a major nucleator at the zonula adherens, while formin is sufficient for F-actin assembly along lateral surfaces. In this regard, previous reports have suggested that different cadherins are responsible for different types of F-actin organizations, which may be mediated, in part, by a different complement of nucleation factors.

It is also important to note that only severe loss of adherens junction proteins causes cell fragility in the epidermis. For example, loss of p120-catenin results in a dramatic reduction in the cortical localization of all adherens junction proteins. However, there are no detectable cell-cell separations in the epidermis of these mice, presumably because desmosomes are sufficient for adhesion under these conditions. Loss of E-cadherin alone also does not result in blistering, partially due to the upregulation of P-cadherin. Combined loss of these 2 cadherin does result in fragility and blistering of the epidermis, demonstrating that these junctions are important, although skin tolerates a significant reduction in their levels. Therefore, while we do not detect any adherens junction defects by any of the assays we have used, it is possible that there are subtle effects that we are missing with our approaches. Examining the role of the Arp2/3 complex in tissues where adherens junction activity is more important for cell adhesion, such as the intestine, should shed additional light on this.

### Tight Junctions

In addition to adherens junctions, cortical F-actin also associates with tight junctions – specialized structures that form a permeability barrier in epithelia. There has been little prior data on the role of Arp2/3 complex in tight junction dynamics and existing data is contradictory. While a couple of studies implicated Arp2/3 activity in the disassembly of tight junctions driven by endocytosis, others have positively implicated Arp2/3 in tight junction function, albeit indirectly. First, the bacterial toxin EspF promotes tight junction disassembly at least in part through mislocalization of N-WASP, Arp2/3 and tight junction proteins. In addition, knockdown of palladin, an Arp2/3 interacting protein, resulted in tight junction defects in Sertoli cells in culture. None of these are especially satisfying in that they did not look directly at effects of loss of Arp2/3 complex components on tight junctions.

In the epidermis, tight junctions form in the differentiated granular layer, forming a water barrier at the outer edge of living cells of this tissue. While loss of ArpC3 did not detectably affect adherens junctions in the epidermis, it did have a dramatic effect on tight junctions. There was a clear decrease in the cortical accumulation of tight junction proteins in the granular layer of the epidermis. In cultured keratinocytes, the phenotype was somewhat distinct in that ZO1 (which can localize to both adherens junctions and tight junctions in cultured cells) did accumulate at junctions but these junctions were disorganized and wavy as compared to their wild type controls. Finally, we found that the functional status of the tight junction was perturbed in ArpC3 null cells, as measured by transepithelial resistance. Therefore, these data established for the first time a requirement for Arp2/3 activity in tight junction function. Again, it will be important to determine whether this is a cell-type specific role or whether simple epithelia also require Arp2/3 for tight junction function.

The underlying mechanism of how the Arp2/3 complex functions in tight junction activity remains unknown. The most obvious explanation is that the F-actin that is linked to tight junctions is assembled by the Arp2/3 complex and this cannot be fully compensated by other mechanisms. An alternative idea is that the Arp2/3 complex is important for the generation of F-actin structures in the cell that allow adequate tension to be generated for tight junction function. We have previously found that tight junction activity is regulated by myosin II-dependent contractility.
**Arp2/3 Complex Function in Cell Migration**

Many epithelial cells have the ability to heal wounds and remodel their organization in response to external or internal cues. In many cases, this is mediated by cohesive sheet migration. Perhaps the most canonical function of the Arp2/3 complex is in promoting cell migration. Early evidence for this included the localization of the Arp2/3 complex to the leading edge and the presence of branched actin networks in lamellae that closely resembled those formed by the Arp2/3 complex in vitro. Recent work in cultured cells has supported a role for the Arp2/3 complex in migration, though it is not essential for this process. Migration tends to be slower and less directional, but cells, including keratinocytes, still migrate without the Arp2/3 complex. The in vivo functional relevance of Arp2/3 complex in cell migration has been established in some invertebrate tissues, such as the C. elegans hypodermis, but it has still not been functionally tested in mammals. It will be interesting to determine to what extent Arp2/3 complex inhibition affects distinct migratory activities during development, in neurons, immune cells, epithelial sheets and in metastasis.

In agreement with work in other cell culture systems, loss of Arp2/3 complex activity in cultured keratinocytes resulted in migration defects both when cells migrate singly or as a cohesive sheet. During epidermal development keratinocytes do not undergo amoeboid crawling movements. Loss of focal adhesion proteins that are important for cell migration, such as FAK, do not result in dramatic developmental phenotypes. That said, like in other cultured cells, loss of Arp2/3 complex activity in keratinocytes resulted in migration defects both when cells migrate singly or as a cohesive sheet. It will be interesting in the future to determine whether focal loss of Arp2/3 in adult skin causes cell autonomous (or non-autonomous) migration defects during wound healing.

**Novel Functions for the Arp2/3 Complex in the Epidermis – Growth Control and Differentiation**

We began our examination of the ArpC3 null epidermis with a focus on known cellular roles of the Arp2/3 complex, such as cell shape, polarity, and adhesion. Clearly from the discussion above, this mouse has taught us both that 1) predicted roles for the Arp2/3 complex were not required in this tissue, and 2) loss of the Arp2/3 complex resulted in unexpected defects not predicted by cell culture work. Two of the most surprising phenotypes that we found upon loss of ArpC3 in the epidermis were an increased number of proliferating cells and a defect in the normal differentiation of this tissue. The increased rate of proliferation was likely due, at least in part, to an increase in pro-mitogenic signaling. We found an increase in the transcript levels of mRNA’s encoding epidermal growth factor receptor ligands, and activation of known downstream pathways, including Jun phosphorylation. These effects appear to occur secondary to the defects in tissue differentiation as isolated keratinocytes in culture did not demonstrate these alterations. Another recent report has identified non-autonomous effects upon loss of Arp2/3 complex activity in fibroblasts, however, in that case the effects were evident in cultured cells. While there were similarities in general between that study and ours (i.e. the increased expression of a number of secreted factors), the specifics were different (i.e., in fibroblasts NF-kB was upregulated, however, there is no evidence for this in keratinocytes). This reiterates the importance of cell type and status in determining the response to loss of Arp2/3 complex activity. In addition to the changes in EGFR ligands, we also noted an increase in the expression of a number of YAP1 target genes, including Cyr61, CCRN41 and CTGF. YAP1 is a transcription factor that acts as a downstream component of the Hippo signaling pathway, which regulates proliferation and organ size. In the skin, gain of function studies have demonstrated that YAP1 hyperactivation results in increased proliferation and differentiation defects, leading to tumorous growths. In addition to its canonical role in the Hippo pathway, YAP1 is also a downstream component of pathways that sense mechanical properties of the cell, including matrix rigidity, cell-size and spreading, cell-cell adhesion and cytoskeletal organization. The molecular pathways linking these phenomenon to YAP1 activity are still poorly understood. In most cases, however, activity is regulated to a significant extent by the localization of YAP1 which can be sequestered in the cytoplasm, or active in the nucleus. In intact epidermis and in cultured keratinocytes, loss of ArpC3 resulted in both an increase in cells with nuclear YAP1 and an increased association of YAP1 with its transcriptional coactivator TEAD. This aberrant activation was necessary for the differentiation defects in ArpC3 mutant epidermis as inhibition of YAP1 resulted in a rescue of these phenotypes, though it had no effect on the tight junction defects. Therefore, our work uncovered a previously unrecognized role for the Arp2/3 complex in regulating YAP1 activity in the epidermis.

This raises the very interesting, and unanswered question, of what cellular changes are sensed upon loss of ArpC3, and how these changes impinge on YAP1 activity. While loss of the cell adhesion protein, -catenin, results in YAP1 activation, we found no evidence for a defect in adherens junctions, as discussed above. Two possible other changes are: 1. a change in cortical tension or 2. a change in F-actin organization. There were clear alterations in F-actin organization in mutant cells, including an increase in the number of stress fibers, and changes in the localization of the actin binding protein -actinin and in the levels/localization of phospho-myosin light chain. However, the current resolution of F-actin organization in tissue samples has not allowed us to definitively describe the differences in mutant skin. As opposed to cultured keratinocytes which are large and flat, keratinocytes in tissue are cuboidal and have a very small cytoplasmic volume, making analysis of F-actin organization difficult. Cultured cells also seemed to be under increased tension, as we saw frequent snapping loss of adhesions in time-lapse images of keratinocytes. However, this did not translate into a statistically significant difference in traction forces as measured by traction force microscopy (Henry Foote, Terry Lechler, unpublished data). Therefore, the specific alterations in F-actin organization associated with altered YAP1 activity remain unknown.
Our finding is not the first implicating Arp2/3 complex in cell fate decisions and differentiation. In *Drosophila* sensory organ precursors, Arp2/3 is required for Notch dependent differentiation. In this context, it appears that formation of a specific actin rich structure and vesicle trafficking to that structure are lost in Arp2/3 mutants. Thus, there are diverse and cell-type specific roles for Arp2/3 complex in differentiation, emphasizing the need to study this complex in in vivo settings.

**Summary**

There is clearly a lot of fundamental cellular biology and physiology left to be learned by studying the functions of the Arp2/3 complex in diverse tissues. In addition to these genetic approaches, it is becoming increasingly important to develop novel tools and methodologies/technologies to image cytoskeletal organization and dynamics in intact tissues. In combination, this will allow future breakthroughs in cytoskeletal control of tissue architecture and function.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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