IDENTIFYING AND ANALYZING THE ROLES OF CDC42 DURING MAMMARY GLAND DEVELOPMENT AND TRANSFORMATION

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IDENTIFYING AND ANALYZING THE ROLES OF CDC42 DURING MAMMARY GLAND DEVELOPMENT AND TRANSFORMATION

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The small GTPase Cdc42 is an essential signaling molecule in multiple cellular processes, including proliferation, migration, division and apoptosis. The overexpression of Cdc42 is found in certain breast carcinomas, bringing to question its roles in normal mammary cell function and during breast cancer progression. In this thesis, I have examined the roles of Cdc42 during mammary gland development, and then analyzed the effects of overactive Cdc42 signaling on mammary epithelial cells (MECs). To accomplish this, I used an *in vivo* mouse model as well as primary cell culture systems.

In the initial study, the conditional-knockout of Cdc42 in the epithelia of adult mouse mammary glands resulted in the altered cellular localization of Par complex members, as well as E-cadherin. These changes accompanied a disorganization of the epithelial cells within the mammary gland, and led to insufficient lactation. This loss-of-function mouse model showed that Cdc42 is essential for the proper maintenance of both apical-basal cell polarity and E-cadherin-based cell-cell junctions in the adult mammary gland. These results raised further interest concerning the roles Cdc42 may have in breast carcinoma development, in which proper apical-basal cell polarity and cell-cell communication are commonly lost.

I next examined the effects of aberrant Cdc42 signaling in primary MECs by utilizing the constitutively-active Cdc42[F28L] mutant. In a monolayer culture system, Cdc42[F28L]

stimulated the formation of actin-based stress fibers, and gave rise to multi-nucleated cells, while in a three-dimensional model system it drove the primary MECs toward an invasive phenotype in an IQGAP1-dependent manner. The primary MECs expressing Cdc42[F28L] lost the proper localization of E-cadherin at cell-cell contacts and no longer formed normal, hollowed alveolar lumens, but instead began to abnormally fill the luminal space and invade out into the surrounding environment.

A common phenotype exhibited by these model systems was the proper maintenance of E-cadherin-based cell-cell contacts between mammary epithelial cells. Interestingly, both the deletion of Cdc42 and its constitutive activation resulted in abnormal E-cadherin expression and localization within mammary epithelial cells. IQGAP1 is likely the primary Cdc42 effector responsible for these phenotypes, as it can both regulate E-cadherin stability at cell-cell junctions as well as bundle microtubules at the leading edge of invasive cells. These studies suggest IQGAP1 is a critical signaling effector of Cdc42 in adult mammary epithelial cells and that, when not properly regulated, it can shift the epithelia toward an invasive phenotype.

BIOGRAPHICAL SKETCH

The author was born in Coudersport, Pennsylvania, a small town surrounded by wilderness and farms. Spending much of his childhood in the forests, fields and streams of northern Pennsylvania, he developed a fascination and deep appreciation for the natural world. This affection grew during his collegiate years as a biology student at The Pennsylvania State University, where courses in evolution, genetics and biochemistry brought a deeper understanding to how the natural world functions. While initially considering graduate school immediately after college, a job offer in Maine drew him to another location surrounded by beautiful nature. However, after several years at this position, he felt disconnected from something he loved about his collegiate experience, which was the continual learning found within academia. This led him to seek a position as a research technician in Richard Cerione's laboratory at Cornell University. Soon after beginning this work, he then entered as a graduate student in the department of Molecular Medicine, where his research combined several aspects of his interests in genetics, development and disease. He intends to continue research within these fields during his postdoctoral position, after which he will seek faculty positions within academia.

To my wonderful family, past and present

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LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
aPKCζ	Atypical protein kinase c isoform ζ
Arp2/3	Actin-related protein 2/3
B-Cat	Beta catenin
Cbl	Casitas B-lineage lymphoma
CC3	Cleaved caspase-3
CCKO	Cdc42 conditional knockout
Cdc42	Cell division cycle 42
CIP4	Cdc42 interacting protein 4
CK8	Cytokeratin 8
CLIP-170	CAP-GLY domain containing linker protein 170
Cool-1	Cloned out of library 1
Cre	Cyclization recombinase
DAPI	4’,6-diamidino-2-phenylindole
Dbl	Diffuse B-cell lymphoma
DCIS	Ductal carcinoma in situ
E-Cad	Epithelial calcium-dependent adhesion molecule
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase

Flox	Flanked by loxp
GAP	GTPase activating protein
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HA	Human influenza hemagglutinin
IQGAP1	IQ-motif containing GTPase activating protein 1
JNK	c-Jun N-terminal kinase
LGL	Lethal giant larvae
LoxP	Locus of X-over P1
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
mDia	Mammalian diaphanous-related protein
MEC	Mammary epithelial cell
MET	Mesenchymal to epithelial transition
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
mTOR	Mechanistic target of rapamycin
N-Cad	Neural calcium-dependent adhesion molecule
PAK	p21-activated kinase
Par3	Partitioning-defective 3

Par6	Partitioning-defective 6
PI3K	Phophoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homolog
Rac1	Ras-related C3 botulinum substrate 1
Ras	Rat sarcoma
Rho	Ras homology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMAD 2/3	Homolog of *Drosophila* mothers of decapentaplegic
Src	Sarcoma
STAT5	Signal transducer and activator of transcription 5
TGFα	Transforming growth factor alpha
TGFβ1	Transforming growth factor beta-1
WAP	Whey acidic protein
WASP	Wiskott-Aldrich syndrome protein
ZO-1	Zona occludens 1

Chapter 1

Introduction

Small molecular-weight guanosine triphosphatases (GTPases) transduce a diverse array of cellular signals that affect cell division, migration, differentiation, and apoptosis (1–3). Cdc42 (for Cell Division Cycle 42) is a member of the Rho subgroup within the Ras superfamily of GTPases, and was first discovered as an essential gene in *Saccharomyces cerevisiae*, where it functions at actin-dependent, polarized budding sites of the plasma membrane to allow the establishment of cell polarity and division (4). In the absence of Cdc42, yeast cells were shown to grow larger yet remain unbudded (5). At roughly the same time, the human homolog, initially named Cdc42Hs, was discovered when searching for novel signaling partners for the epidermal growth factor receptor (EGFR) (6). Further studies extended our understanding of the actions of Cdc42 in mammalian cell function, where it was shown to play essential roles not only in the polarization of epithelial cells, but also in their migration, intracellular trafficking, differentiation, metabolism and division (7–11). Considering the wide array of mammalian cellular functions now associated with Cdc42, some of which are found to be deregulated in disease states (12–14), a key question arises in understanding how higher-evolved cells orchestrate Cdc42 signaling to perform their various tasks in functional tissues. This question is relevant to the study of tumorigenesis, particularly when considering that Cdc42 is found to be overexpressed in human invasive breast

ductal carcinoma (12), and that the expression of a hyper-active mutant of Cdc42 causes NIH3T3 fibroblasts to attain hallmark characteristics of cancer (15).

Cdc42 signaling is tightly regulated in cells

The expression of Cdc42 is important for dictating appropriate cell signaling, but the mere presence or absence of the translated Cdc42 protein is not the only means by which downstream signaling is controlled. One level of control over Cdc42 signaling is through a binary switch-like mechanism that turns the downstream signaling "on" or "off". This switch-mechanism of activity provides a secondary level of control over the activation of downstream signals via the nucleotide binding status of Cdc42. When Cdc42 binds to guanosine triphosphate (GTP), it is considered "active" and has enhanced binding potential to downstream effector molecules. Cdc42's intrinsic GTPase enzymatic activity will eventually hydrolyze the GTP into guanosine diphosphate (GDP), which returns Cdc42 to an "inactive" state that no longer favors effector-binding. While the intrinsic rate of GTPase activity of Cdc42 is quite low, it can be greatly accelerated by the binding to Cdc42 of a GTPase activating protein (GAP) (16). When a GAP binds to GTP-bound Cdc42, a structural shift occurs that allows the hydrolytic cleavage of a phosphate group from the GTP, and the resultant GDP that occupies the nucleotide binding pocket lowers the potential for binding downstream effector molecules. However, when a guanine nucleotide exchange factor (GEF) binds to GDP-bound Cdc42, it destabilizes the binding of nucleotide to Cdc42, resulting in dissociation. This nucleotide-free Cdc42 is then likely to bind to GTP (which is, on average, at a ten-fold higher concentration than GDP in the cytoplasm of the cell), thereby activating downstream signaling (17). GAP proteins can therefore be thought of as negative regulators of downstream Cdc42 signaling, and GEF proteins as positive regulators. These classes of molecules that affect the activity of Cdc42 become extremely relevant to cancer biology, as a hyperactive,

truncated mutant of a Cdc42 GEF is found in diffuse b-cell lymphoma (18), while a Cdc42 GAP is poorly expressed in certain liver cancers (19). In each of these cases, the control over Cdc42 activity and its downstream signaling is altered in cancer cells.

Another class of Cdc42-regulatory proteins has emerged from studies of Rho GTPases, and they are referred to as guanosine nucleotide dissociation inhibitors, or GDIs. GDIs have the ability to facilitate the dissociation of Cdc42 from the plasma membrane, and can also inhibit GDP exchange as well as GTP hydrolysis (20). This allows GDIs to have a unique level of control over not only the nucleotide binding state of Cdc42, but also its localization in the cell. Together, these GEFs, GAPs and GDIs provide a mechanism for cells to orchestrate Cdc42 signaling and localization with high fidelity, allowing the expressed Cdc42 protein to be used in multiple cellular processes (Figure 1.1).

Cdc42 modulates the cell cytoskeleton through a variety of effectors.

The stimulation of G-protein-coupled receptors, receptor tyrosine kinases and integrins can all lead to the activation of GEFs for Cdc42 (21). Then, once Cdc42 is GTP-bound, a wide array of downstream binding partners, or effectors, can be activated. While Cdc42 was initially shown to be essential for the regulation of budding site selection in yeast (4) , thereby allowing polarized cell growth, it was later found that Cdc42 is also a regulator of the mammalian cell cytoskeleton through its potential binding to several effector molecules, including the Wiskott-Aldrich Syndrome protein (WASP), the mammalian Diaphanous-related (mDia) formin, the scaffold protein IQ-Motif Containing GTPase Activating Protein 1 (IQGAP1), and the p21-Activated Kinases (PAKs). Given the accumulating evidence highlighting the critical impact of the cytoskeleton on various cell functions and survival, the ability of Cdc42 to have multiple

Figure 1.1. The regulation of Cdc42 signaling to downstream effectors and its cellular effects. GTP-bound Cdc42 can act through a variety of effectors to affect multiple cellular processes, including cytoskeletal dynamics, differentiation and mitosis. The tight regulation of Cdc42 signaling activity is attained through the actions of guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). When bound to Cdc42, GEFs induce the release of GDP, allowing subsequent binding of GTP, thus activating downstream signaling. When bound to Cdc42, GAPs induce GTP hydrolysis, thereby ceasing downstream signaling. GDI-binding to Cdc42 causes dissociation from the membrane, and can inhibit GDP exchange as well as GTP-hydrolysis.

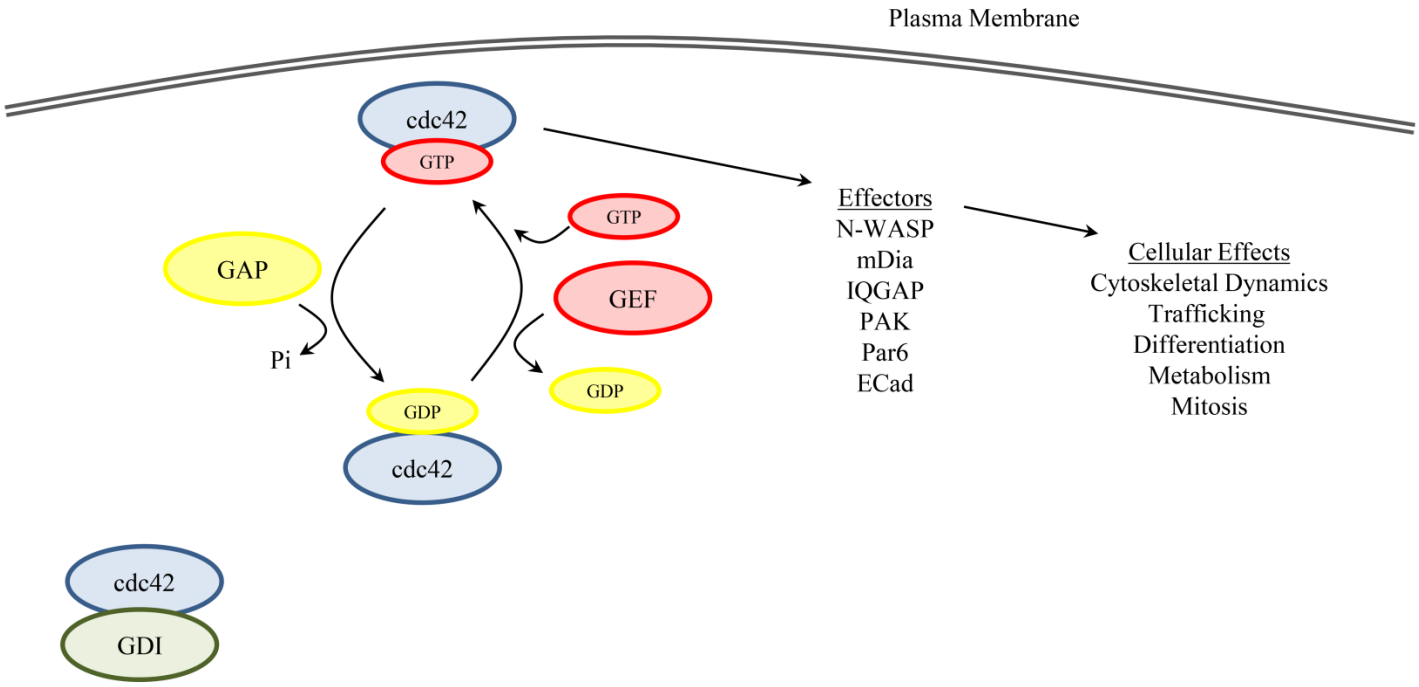
Plasma Membrane
cdc42
GTP
GTP
GAP
GEF
Pi
GDP
GDP
cdc42
cdc42
GDI
Effectors
N-WASP
mDia
IQGAP
PAK
Par6
ECad
Cellular Effects
Cytoskeletal Dynamics
Trafficking
Differentiation
Metabolism
Mitosis

Figure 1.1

inputs into cytoskeletal dynamics contributes to the multiple roles played by this small GTPase in both normal development and disease.

In studies that further examined mechanistically how Cdc42 is able to modulate the cell cytoskeleton, it was found that by binding to WASP, activated Cdc42 released an auto-inhibitory structural fold of WASP, thereby allowing it to interact with the Actin-Related Protein 2/3 (Arp 2/3) complex to initiate actin assembly into branched filaments of the cytoskeleton (22, 23). GTP-bound Cdc42 was also shown to bind to mDia, releasing an auto-inhibitory conformation in the formin and inducing its actin filament assembly activity, such as the linear actin filaments found in filopodia and stress fibers within the cell (24). Upon binding to the N-terminal regulatory domain of PAK family members, activated Cdc42 again releases an auto-inhibitory structural fold in these effectors, which then allows PAK to phosphorylate and inhibit Myosin Light Chain Kinase (MLCK) at the cell membrane, resulting in the promotion of lamellipodia formation (25, 26). In a highly-esteemed and critical study from Alan Hall's laboratory, the microinjection of the constitutively-active Cdc42(V12) mutant into Swiss 3T3 cells results in the formation of filamentous actin-rich filopodial extensions along the cell periphery, highlighting the potential of GTP-bound Cdc42 to drastically affect actin cytoskeletal dynamics (27).

IQGAP1, although not a small GTPase GAP as its name suggests, does act as an effector of Cdc42 and interestingly, when GTP-bound Cdc42 binds to IQGAP1, this interaction inhibits the hydrolysis of GTP (28), thereby leading to larger pools of activated Cdc42 (29). IQGAP1 is a scaffolding protein with binding sites for actin and it has been shown to crosslink actin filaments, thereby potentiating the formation of filopodia (30, 31). The expression of IQGAP1 in mammalian cells has been shown to increase the cellular levels of GTP-bound Cdc42 and produce actin-rich microspikes at the plasma membrane (29). It has also been shown that, when not bound to Cdc42,

IQGAP1 can bind directly to E-Cadherin and β-catenin and that this binding weakens the interaction of the actin cytoskeleton to adherens junctions between cells, resulting in increased degradation of E-Cadherin and therefore weakened cell-cell contacts (32, 33). This dual-effect of IQGAP1 can be seen as a critical component of the transition of static, organized epithelial cells into a migratory phenotype.

Taken together, these data place Cdc42 as a master regulator of actin-cytoskeletal dynamics, upon which a substantial amount of cellular processes rely. Cdc42-signaling has also been shown to directly affect a range of cellular processes other than cytoskeletal modulation, including cell adhesion (34, 35), migration (36), apoptosis (37–39), cell-cycle progression (40–42) and polarity establishment (43–46). Collectively, these studies of Cdc42 function in cell culture models have provided enormous insight into the myriad of cell signals that are affected by this GTPase, and have laid the framework for further investigating its roles *in-vivo*.

Roles of Cdc42 in vivo

Cdc42 has been shown to be an essential gene for higher eukaryotic life (2, 47), which relies on cells that must coordinate with one another to form functional tissues. In *C. elegans*, Cdc42 is essential for the maintenance of the cellular asymmetry that allows proper mitotic spindle orientation and mitosis (48). During *Drosophila melanogaster* development, Cdc42 plays a role in the establishment of apical and basolateral cell polarity (49), a critical step which allows the organization of cells during morphogenesis, and affects epithelial cell cohesion during tissue formation. As studies moved into mammals, it was shown that the complete deletion of Cdc42 in the mouse (*Mus Musculus*) resulted in disorganized cells and a lack of primary ectoderm formation which caused lethality at embryonic day 6.5 (50). Considering the multiple signaling networks that can be affected by Cdc42, it is logical that its expression is absolutely essential for mammalian

embryogenesis. This embryonic-lethal phenotype prompted researchers to employ conditional knockout strategies to study Cdc42 in the mouse. Use of the Cre/loxp system has provided this opportunity to study the roles of Cdc42 in selected mouse tissues of interest, and furthered our understanding of Cdc42 signaling by illustrating a physiological perspective to the roles of this small GTPase.

In the heart, the loss of Cdc42 expression resulted in hypertrophic cardiac cell growth (51). Given the prior studies showing that Cdc42 can promote growth signaling in cells (52, 53), it was surprising to see that the loss of its expression resulted in hypertrophic cell growth. This study showed that, in the heart, Cdc42 signaling activates c-Jun N-terminal kinase (JNK), which provides a protective and anti-hypertrophic signal in cardiomyocytes which have calcineurin-NFAT activity induced by stress (51). However, this was not the only unexpected phenotype accompanying the conditional deletion of Cdc42 in mouse tissues. In neural progenitor cells, Cdc42 was found to be responsible for the establishment of apical-basal polarity, but it did not affect the normal progression of the cell cycle. Interestingly, the conditional deletion of Cdc42 in this model resulted in an abnormally high number of basal progenitor cells within the tissue, due to an inability of the cells to migrate appropriately based on proper cell polarity (54). Pancreatic development in the mouse was also shown to rely on Cdc42 signaling, again via the proper establishment of cell polarity during the morphogenesis of this tubulous organ. Loss of Cdc42 expression during the formation of the pancreas resulted in an abnormal organization of the cells within the organ, and a consequent alteration of the differentiation status of the cells (55). Deletion of Cdc42 in skin keratinocytes of the mouse also resulted in a change of cell fate, and in the skin this was shown to be caused by alterations in β-catenin regulation (56). Aberrant deposition of basement membrane components was also found at the dermal-epidermal junction of these mice

(57), suggesting that Cdc42 also contributes to the maintenance of the extracellular environment by keratinocytes in the skin. In the intestinal epithelia of the mouse, the deletion of Cdc42 affected several morphogenetic processes, resulting in hyperplasia, abnormal epithelial permeability, irregular migration and altered cell differentiation (10). These studies, along with many others, have begun to illuminate multifaceted roles played by Cdc42 not only at the cell-signaling level, but also at the tissue and/or physiological level where cells must communicate and coordinate with one another (Figure 1.2). By interfering with the critical ability of cells to sense and respond to their surrounding tissue environment, Cdc42 conditional-deletion can ultimately result in an altered cell-fate and functional status, rendering the tissue ineffective.

C*dc42 regulates epithelial cell polarity*

Many of the studies of Cdc42 *in-vivo* share a common denominator in the resultant phenotypes, which is an altered cell polarity that leads to tissue-specific defects (8, 10, 49, 55, 58). This brings into focus the importance of understanding how epithelial cells sense surrounding growth factors, basement membranes, and other cells within the tissue to regulate their processes. The evolution of mammalian epithelial cells has resulted in the differentiation of various exquisite tissues and organs. Epithelial cells line the exterior and interior surfaces of mammalian bodies, and thereby provide a functional barrier between tissues of the body, or between the tissue and its external environment. To accomplish this, the epithelial cells must establish and maintain a synchronous barrier by making contacts with one another in single or multiple layers, such that selective diffusion may take place across the formed barrier. This allows for the passage of some molecules through the epithelial sheets, for example nutrients or hormones, but not others such as

Figure 1.2. Cdc42 plays various roles *in vivo*. Multiple essential roles for Cdc42 have been identified in several *in vivo* model systems. Cdc42 has been shown to affect cell polarity, adhesion and mitosis in *C. elegans* and *D. melanogaster*, and plays roles in cell fate, polarity and growth in various tissues in *M. musculus*.

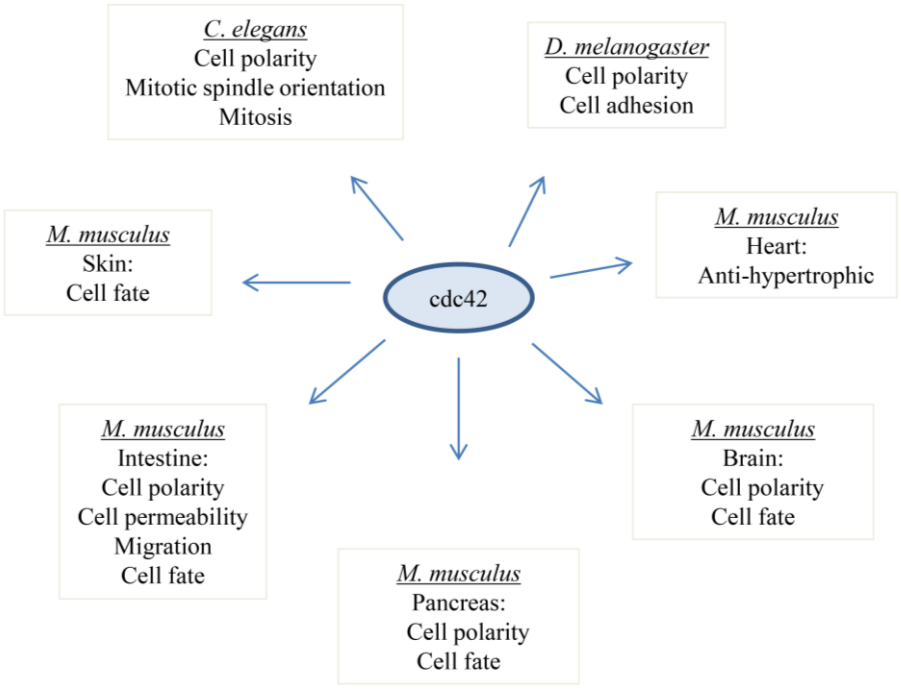
C. elegans
Cell polarity
Mitotic spindle orientation
Mitosis
D. melanogaster
Cell polarity
Cell adhesion
M. musculus
Skin:
Cell fate
cdc42
M. musculus
Heart:
Anti-hypertrophic
M. musculus
Intestine:
Cell polarity
Cell permeability
Migration
Cell fate
M. musculus
Brain:
Cell polarity
Cell fate
M. musculus
Pancreas:
Cell polarity
Cell fate

Figure 1.2

toxins or waste. These cell-to-cell contacts, as well as the epithelial cells' attachment to a basement membrane, allow single cells to behave in an orchestrated manner to form functional tissues. The establishment and maintenance of these associations between differentiated epithelial cells have been shown to exert remarkable effects on cell growth, survival, migration and invasion (59, 60), all of which are necessary for tissue-level functionality. Alterations in these processes in epithelial cells have become hallmarks of cancer and, as approximately ninety percent of human cancers are derived from epithelial cells (carcinomas), understanding the molecular mechanisms associated with epithelial architecture and function becomes focal to developing higher-efficacy cancer therapies.

One way in which cells associate with each other is through adherens junctions. Adherens junctions represent a primary form of cell-to-cell adhesion, and are established by the homotypic binding of cadherins (calcium-dependent adhesion molecules) (61). In mammalian epithelial cells, the extracellular domain of the transmembrane protein epithelial cadherin (E-Cadherin) is able to bind to another E-Cadherin protein in a neighboring cell, while the intracellular domain of E-cadherin can anchor to the actin cytoskeleton via α-catenin and β-catenin (62, 63). In this way, single epithelial cells can join together their cytoskeletons to form a tissue-level organization and function. Multiple associations between E-Cadherin molecules form zipper-like clusters between cells, and these clusters are crucial for the formation of epithelial cell layers (64). As its name suggests, E-Cadherin's homophilic interactions are dependent on calcium binding to a linker region between the extracellular domains, such that the depletion of calcium leads to the endocytosis of E-Cadherin molecules from the plasma membrane (65–67). These endocytosed molecules are then either recycled back to the plasma membrane to strengthen adherens junctions, or they are targeted for lysosomal degradation which then leads over time to weakened adherens

junctions. In this sorting of E-Cadherin molecules, adherens junctions are consistently monitored and modulated to allow tissue growth and organization, as well as for tissue healing after injury (68, 69). Interestingly, the loss of E-Cadherin expression is correlated to cancer progression (70–73), and the expression of E-Cadherin in certain cancers suppresses their invasive and metastatic potential (70, 74).

The activity of the epithelial growth factor receptor (EGFR) has been shown to regulate the stability of E-Cadherin-based adherens junctions, by activating the non-receptor tyrosine kinase Src (Sarcoma) (75). Activated Src is then able to phosphorylate E-Cadherin, leading to subsequent ubiquitination by Hakai and lysosomal targeting (76, 77). Cdc42 has been shown to be instrumental in this process by potentiating the EGFR-mediated activation of Src, leading to the increased lysosomal targeting and degradation of E-Cadherin molecules (75). Activated Cdc42 potentiates EGFR signaling by binding to Cool-1 (Cloned-out-of-library), which binds to the E3 ubiquitin ligase Cbl (Casitas B-lineage lymphoma), thereby preventing Cbl from catalyzing EGFR ubiquitination for subsequent degradation (78). This interaction between Cbl and EGFR is critically important for proper cell and tissue growth, and the deletion of c-Cbl in the mouse results in hyperplasia (79). Interestingly, the loss of E-Cadherin molecules at adherens junctions has also been shown to release p120-catenin from the cell membrane and into the cytoplasm (80, 81). Once cytoplasmic, p120-catenin has the potential to activate Cdc42 via the GEF Vav2 (82). Taken together with the previously-mentioned roles for Cdc42 with IQGAP1, these data collectively suggest a dynamic mode of steady-state monitoring of E-Cadherin and EGFR by Cdc42 in epithelial cells (Figure 1.3).

Within our bodies, many organs contain hollow tubules and spheres that are lined by a

Figure 1.3. Cdc42 affects E-cadherin stability through multiple pathways. E-cadherin (E-Cad) molecules bind together at adherens junctions between cells that, when bound to the cytoskeleton via catenin family members, form a communication network between epithelia. The binding of IQGAP1 to b-catenin inhibits E-cadherin binding to b-catenin, and consequently the cytoskeleton, thus destabilizing E-cadherin at the cell membrane. Cdc42 can prevent this process by binding to IQGAP1, which inhibits IQGAP1-b-catenin interactions and thus helps stabilize E-cadherin at cell-cell junctions. P120-catenin, when not bound to the cadherin-catenin complex, can activate the Cdc42 GEF Vav2. EGFR activation can cause Src-mediated phosphorylation of E-cadherin molecules which, followed by ubiquitylation by Hakai, can lead to lysosomal degradation of E-cadherin. Cbl, a negative feedback regulator of the EGFR, can indirectly mediate this process of E-cadherin phosphorylation by Src. Cdc42, by binding to the Cool1, a negative regulator of Cbl, can potentiate EGFR signaling and thus E-cadherin phosphorylation by Src. Together these processes represent a complex network of adherens junction maintenance through Cdc42 signaling.

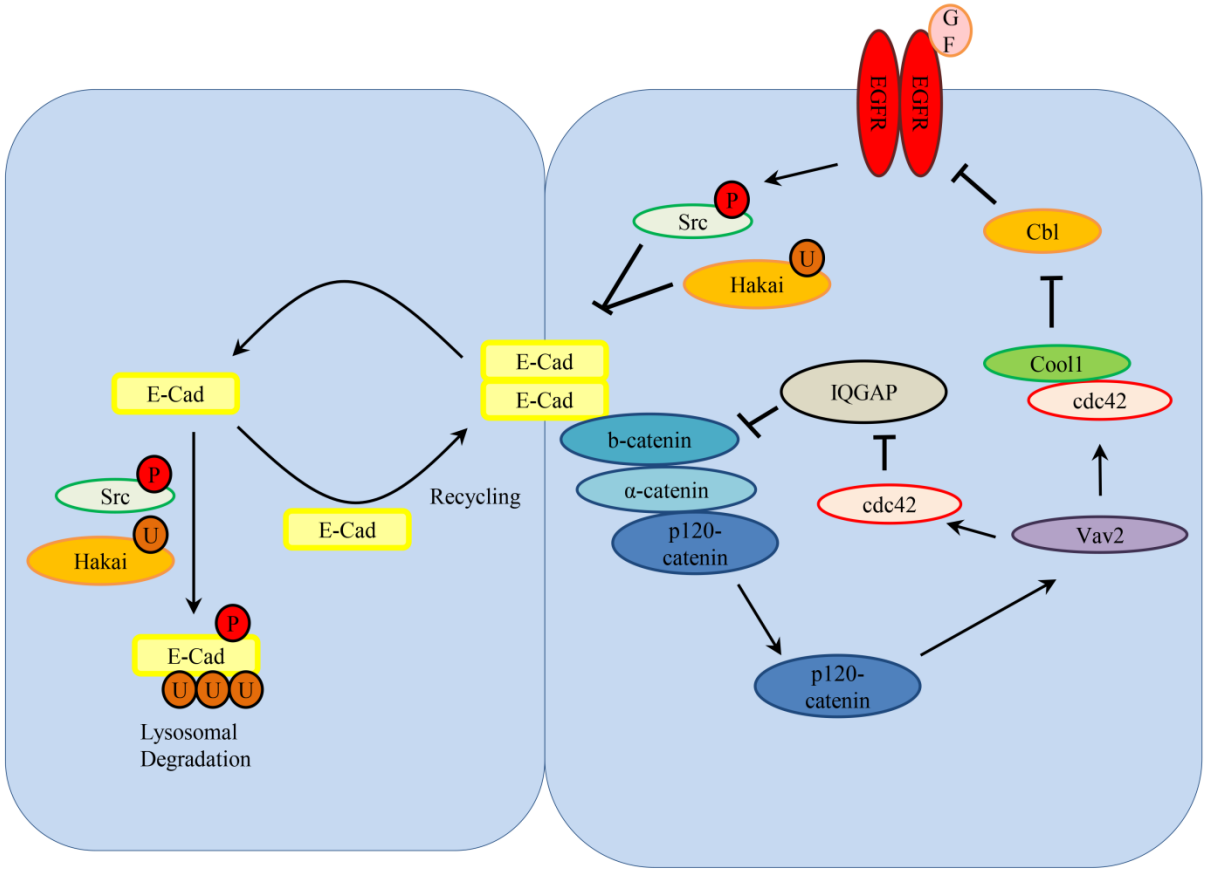
G
F
EGFR
EGFR
P
Src
U
Hakai
Cbl
Cool1
cdc42
IQGAP
E-Cad
E-Cad
E-Cad
b-catenin
α-catenin
p120-
catenin
cdc42
Vav2
P
Src
U
Hakai
Recycling
E-Cad
P
E-Cad
U U U
Lysosomal
Degradation
p120-
catenin

Figure 1.3

layer of polarized epithelial cells that establish and maintain these adherens junctions. The functions of these organs are dependent on compartmentalizing molecules, a process that relies heavily on the formation of barriers by epithelial cell linings. To form and maintain these barriers, some epithelial cells must establish what is known as an apical-basal polarity, such that a basal surface is established near the basement membrane formed within the tissue, and an apical surface establishes on the opposing side of the cell where a central lumen exists (Figure 1.4A). This type of cellular architecture allows for the vectorial secretion of fluids and molecules, such as saliva or milk, into the central lumen, but not into the surrounding cells or tissues. The polarized distribution of ion channels, transporters and pumps within the plasma membrane generates an apical-basal sodium gradient that helps to achieve this vectorial movement of molecules and solutes. Like adherens junctions, the maintenance of this type of epithelial apical-basal polarity is lost in carcinoma development, and is thought to be a powerful regulator of cell growth and invasive migration (83–87). Apical-basal polarity is established and maintained in epithelial cells primarily by three evolutionarily-ancient cellular machineries: the polarized trafficking machinery, which sorts and delivers proteins and lipids to particular cellular locations; the domain-identity machinery, which employs highly-conserved polarity proteins and lipids to generate tight junctions between the cell's apical and basolateral domains; and the three-dimensional organization machinery, which receives and coordinates extracellular signals to control cell growth and cytoskeletal organization.

Along with adherens junctions providing cell-to-cell sensing, apical-basal polarized epithelial cells in vertebrates also form tight junctions between one another, localized toward the apical surface. Tight junctions employ molecules like claudins and occludins which form strands

Figure 1.4. Epithelial organization within ductal tissues relies on apical-basal polarity, which is maintained by polarity complexes within the cell. A) Ducts within tissues are comprised of epithelial cells (blue) that organize together to form a central lumen (upper diagram), which requires apical-basal cell polarity (lower diagram). The apical surfaces of the epithelial cells together form the central lumen, while the basal surfaces adhere to the basement membrane (red). B) Apical-basal polarity is maintained within the epithelial cell by mutual-exclusion actions of polarity complexes: the Par, Crumbs and Scribble complexes. Mutual exclusion is achieved by members from these complexes phosphorylating each other, for example aPKC can phosphorylate and exclude Lgl from the apical surface of the cell and, conversely, Lgl can phosphorylate and exclude Par and Crumbs complex members from the basal surface.

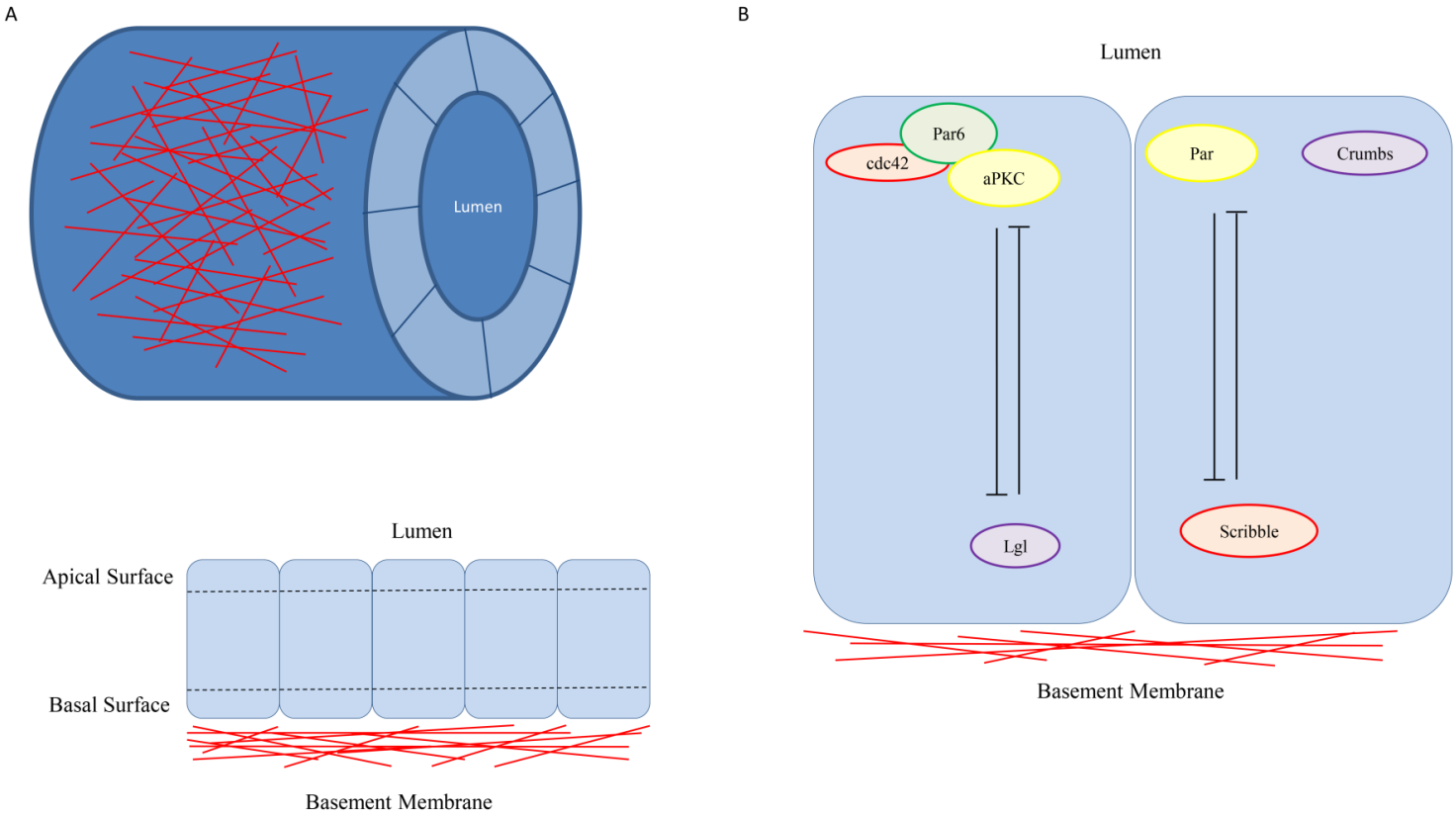
A
Lumen
Lumen
Apical Surface
Basal Surface
Basement Membrane
B
Lumen
Par6
cdc42
aPKC
Par
Crumbs
Lgl
Scribble
Basement Membrane

Figure 1.4

between the cells and anchor to their cytoskeletons via binding to zona occludens 1 (ZO-1) molecules (88). The tight junctions provide a paracellular barrier function between the apical surfaces of cells, allowing the passage of select solutes and molecules, and they mark the division between the apical and lateral surfaces of epithelial cells. While many molecules are part of the tight junction, its assembly and maintenance have been shown to be dependent on the concerted actions of Par3 (Partitioning-defective 3), Par6, and Cdc42 (89, 90).

Three main molecular complexes, referred to as the Par, Crumbs, and Scribble complexes, are associated with the establishment and maintenance of epithelial apical-basal polarity (91, 92). In mammalian epithelia, these complexes can work in a fascinating mutual-exclusion relationship with one another, such that members of the Par complex exclude members of the Scribble complex from the apical cell surface, and conversely, members of the Scribble complex exclude members of the Par complex from the basal or lateral cell surfaces (93) (Figure 1.4B). This mutual-exclusion feedback mechanism allows epithelial cells to form an apical-basal polarity with distinct enzyme-activity zones. Along with the multiple roles shown for Cdc42 in organizing the cytoskeleton of the cell, it has also been shown that Cdc42 can directly affect the polarization of cells (91), i.e. the front-rear morphology found in migrating cells, as well as the apical-basal morphology found in some differentiated epithelia. The groups of Cdc42 effectors that are involved in acquiring these two separate types of polarity are surprisingly similar, underscoring their importance in a disease such as cancer, in which invasive cancer cells that give rise to metastases have shifted from the differentiated epithelial cell apical-basal polarity into an invasive front-rear polarity.

The core of the apically-localized Par complex consists of Par3, Par6, and atypical protein kinase C (aPKC) molecules (94, 95). In this complex, Par6 functions in recruiting substrates for, as well as regulating the kinase activity of, aPKC (94, 96). Activated aPKC can phosphorylate

molecules such as Lethal Giant Larvae (Lgl) (97), thereby excluding their attachment and activity at the apical cell surface. This results in the localization and activity of Lgl only at basal and lateral cell surfaces. Here, Lgl performs a similar, but inverse, function to aPKC by excluding Par6 activity at the basal and lateral cell surfaces (49). The concerted activities of these members of the polarity complexes result in a steady-state of apical or basolateral molecular localizations, affecting numerous enzyme activities, trafficking, and membrane lipid composition.

Apical-basal-polarized cells also attain unique phosphoinositide enrichment in their apical and basolateral plasma membranes. The apical surface of an epithelial cell is PIP2-rich due to the actions of PTEN (for phosphatase and TENsin homolog) (98). This PIP2-rich plasma membrane at the apical surface is then capable of recruiting and bringing together molecular complexes that contain Cdc42, its GEFs and effectors (85). Cdc42 has been shown to bind directly to Par6, and this interaction is essential for the apical localization of Par6 (99, 100), which then further leads to the recruitment and activation of aPKCζ. Phosphoinositide 3-Kinase (PI3K) performs an opposing effect to PTEN, in that it can phosphorylate the third carbon of the inositol group of phosphoinositides, converting PIP2 into PIP3. Activated aPKCζ has been shown to impair PI3K signaling, thereby keeping the apical plasma membrane enriched for PIP2. In this way, Cdc42 has effects on the balance of apical and basolateral membrane composition, molecular complex localization and, consequently, epithelial cell apical-basal polarity.

However, the Par polarity complex is not exclusively used by differentiated epithelial cells to establish apical-basal polarity. The same Par complex has been shown to localize at the leading edge of migrating epithelial cells (101–103). Integrin binding and focal adhesion stabilization at the cell's leading edge recruits Cdc42, and again leads to aPKC activation through Par6 (46, 104). In this case, however, activated aPKC then phosphorylates its substrates within range of the

leading edge, one of which is glycogen synthase kinase 3β (GSK3β) (105). When GSK3β is phosphorylated at an inhibitory serine residue, it then dissociates from its binding partner APC (adenomatous polyposis coli). When not bound to GSK3β, APC gains the ability to bind to microtubules at their (+) ends and anchor them at the plasma membrane, allowing polarized migration and a front-rear cell morphology. IQGAP1, an effector for Cdc42 previously mentioned for its effects on E-Cadherin stability at adherens junctions, can also interact with APC, CLIP-170, Rac1 and Cdc42 at the leading edge of a migrating cell (106, 107). This complex is thought to orient and stabilize microtubules at the leading edge to form lamellipodia into which vesicles can be trafficked for further migration and invasion, as well as to provide the microtubule stability that is needed for dynein-dynactin motor complexes to reorient the microtubule organization center and Golgi network toward the direction of migration (108). These critical cellular functions in apical-basal versus front-rear polarization display the multifaceted nature of Cdc42 in cell signaling and emphasize the importance of its proper use in epithelial cells of the human body.

Alternative to the static architecture found in much of the epithelia within our tissues, some epithelial cells must move within a tissue from one location to another. This movement, or migration, allows for the dispatchment of the cell from its former locus and cell-to-cell contacts into a new area of the tissue, where it can then establish a new set of cell-to-cell contacts. This entire process of transdifferentiation can be described in two phases: first an epithelial-to-mesenchymal transition (EMT), and then a mesenchymal-to-epithelial transition (MET). Epithelial cells are distinguished by their ability to adhere to one another and form partitions, or barriers, within the tissue while mesenchymal cells have weak adherence to one another which allows them to be motile within the tissue. The employment of these two processes is critical throughout embryogenesis, organogenesis and adult wound-healing, but the improper

transdifferentiation of cells can also contribute greatly to the pathologies of cancer metastasis (109).

During EMT, several distinct cellular biochemical changes occur which allow the apical-basal polarized cell to break down its contacts to adjacent epithelia and attain mobility with a front-rear (directional) polarization. These changes include transcription factor activation leading to gene and microRNA expression, extracellular matrix (ECM) degradation, and the reorganization of the cytoskeletal network of the cell (110). Previous studies have shown Snail to be a major player during EMT, originally by its ability to repress E-Cadherin expression by binding to 3 E-boxes within the E-Cadherin gene promotor (111), and more recently by its ability to repress the expression of Crumbs3 (112). Interestingly, E-Cadherin and Crumbs have been shown to have tumor suppressing characteristics, and the loss of their expression is linked to human tumorigenesis (113–116) .

In some instances, cell migration requires the breakdown of the basement membrane proteins that line the basal side of the epithelial sheets. Migration through this dense matrix of basement membrane proteins is termed invasion, and requires the invading cell to release matrix metalloproteinases (MMPs) which degrade the extracellular matrix at the leading edge. While epithelial cell invasion occurs at low levels in adult tissues, and therefore MMP expression is low in healthy adults, the aberrant activation of MMP expression and cell invasion can facilitate the metastasis of cancerous cells within the body (117). As Cdc42 has been shown to modulate the cytoskeleton of cells through various mechanisms, its potential effects on migration and invasion in cancer cells have also been examined. The contributions of Cdc42 toward breast cancer cell migration and invasion are not fully understood, however, and recent data indicate that it may play contradictory roles depending on the cancer cell type. One group has shown that the knock-down

of Cdc42 in Hs578T cells, a moderately metastatic breast cancer, inhibited migration and invasion. However, in the same study, using MDA-MB-231 and C3L5 cells, which are highly metastatic breast cancer cell lines, the knock-down of Cdc42 enhanced migration and invasion (118). This group went further to show that the knock-down of Cdc42 in these highly metastatic cell lines led to increased phosphorylation of Protein Kinase C-δ, Protein Kinase A, and extracellular signal regulated kinase 1/2 (ERK 1/2). The overexpression of Cdc42 in these breast cancer cell lines showed the same confliction of results by inhibiting migration and invasion in the highly metastatic cells, but stimulating migration and invasion in the moderately metastatic line.

The second phase of transdifferentiation is an inverse process to the EMT, where a mesenchymal cell transitions back into an epithelial cell. The migrating mesenchymal cell ceases its movement within a tissue and shifts its front-rear morphology into an apical-basal polarized morphology and establishes cell-to-cell contacts via adherens and tight junction formation. This transition completes the transdifferentiation cycle and demonstrates how epithelial plasticity is complex but essential to tissue formation and homoestasis (119). However, cells that lose the proper control over these transdifferentiation programs become detrimental to their surrounding tissues, as is evidenced in the case of metastatic cancer cells. These cancerous cells not only grow abnormally and fail to perform proper tissue functions but, upon undergoing EMT, they are capable of spreading to surrounding tissues, including blood. Once in the blood circulation, this type of cancer cell then has the potential to metastasize to other parts of the body, which has garnered the attention of cancer researchers to better understand the process of EMT for potential therapeutic intervention points.

Cdc42 deregulation in the progression of cancer

Cdc42 is found to be overexpressed in multiple forms of cancer (13, 120–123), including certain invasive ductal carcinomas of the breast. Cdc42 has been shown to promote tumorigenesis in several ways, in particular by activating PAK (124, 125), PI3K (126, 127),and mTOR (mechanistic target of rapamycin) (128–130), as well as by inducing the expression of glutaminase C (131) to alter cell metabolism, growth and cytoskeletal remodeling. Cdc42 also affects the cell's DNA damage response, as evidenced by a study targeting the deletion of Cdc42GAP in mice. By deleting a negative regulator of Cdc42, this group showed that mice had elevated Cdc42 activity accompanied by premature aging characteristics with dampened DNA damage repair (132). Cdc42 has also been shown to play a role in the growth signaling of primary mouse embryonic fibroblasts that have lost the expression of p53, a known tumor suppressor (133). With such a significant array of potential signaling outputs to regulate cell growth and function, the studies of Cdc42 in cancer have become enormous in number, and have underscored the weight of this protein's cellular impacts.

The Cerione laboratory has been heavily involved in studying the mechanisms by which Cdc42 is regulated in the cell, from its activation by GEFs to its de-activation by GAPs and sequestration by GDIs. The proto-oncogene Dbl (diffuse B-cell lymphoma) functions as a GEF for Cdc42, and a truncated mutant lacking the N-terminal 497 residues results in the deletion of an auto-inhibitory motif, thereby resulting in an oncogenic version of Dbl (onco-Dbl) that is capable of hyperactivating Cdc42 (134). Expression of onco-Dbl has also been shown to confer cancer-like properties to NIH 3T3 fibroblast cells (18, 135), prompting questions about the role of Cdc42 during this process. To better understand the cellular effects of excessive Cdc42 signaling, several activating-mutants of Cdc42 were created. In these studies, it was noticed that mutants which enabled the accelerated cycling of nucleotide-binding to Cdc42 were capable of promoting cellular

transformation, as opposed to GTP-hydrolysis-defective mutants that locked Cdc42 in a persistently active, GTP-bound state (136). One of these so called "fast-cycling" mutants, Cdc42[F28L], was shown to be capable of GTP hydrolysis, while exhibiting constitutive GDP-GTP exchange. In a cellular environment, the levels of GTP are approximately ten-fold higher than the levels of GDP, thus enabling the Cdc42[F28L] mutant to bind to GTP following GDP release, thereby increasing the cellular pool of GTP-bound Cdc42. The Cdc42[F28L] mutant was also able to confer onto NIH 3T3 fibroblasts several hallmarks of cancer including the ability to grow in low serum and in the absence of a substratum (15). ERK activity was shown to be elevated in the presence of Cdc42[F28L], as a result of sustained EGFR signaling (78). Interestingly, GTP-hydrolysis-defective mutants of Cdc42, which are dominant-active, do not convey the same characteristics of transformation to NIH 3T3 cells. Rather, these dominant-active Cdc42 mutants appear to be growth-inhibitory or toxic in this cell line (136, 137).

Mammary gland evolution and function

Along with epithelial cells forming the barriers within our body, glandular structures are also composed of epithelial cells, both in the cysts and tubules that form the functional units within glands. In the case of a secretory gland such as the mammary gland, these cysts, or alveoli, undergo rapid expansion upon pregnancy and become high-output milk producing factories upon parturition. Carl Linnaeus originally assigned the Class Mammalia in 1758, and did so primarily based on the presence of the mammary glands that distinguish us from other vertebrates. Since then, approximately 5000 species of mammals have been defined, and are diversified to swim, run, or fly. While epithelial cells emerged in metazoans approximately six-hundred million years ago, the mammary gland is an evolutionarily recent feature derived from the ectoderm and found in the past two-hundred million years of life. The evolution of secretory mammary tissue has provided

a unique advantage to survival, as the components of milk are capable of completely nourishing neonates without any other food source. To produce this complete form of nutrition in copious amounts that can sustain the growth of one, two, five, or even ten nursing youth, enormous quantities of energy are required from the mammalian mother, both for the morphogenesis of the secretory tissue during pregnancy, as well as for the synthesis of large volumes of milk during lactation. The level of required cellular control over growth, mitosis, migration, apoptosis and secretion throughout pregnancy and lactation is staggering to consider, and many elegant studies have elucidated in great detail how the mammary gland has evolved to accomplish this (138–140).

While the virgin mammary gland is composed predominantly of adipocytes with only a rudimentary epithelial tree ingrown from the nipple, the onset of pregnancy will induce the rapid proliferation and expansion of the epithelia, particularly the alveolar luminal cells that will synthesize milk proteins and fats. By the time-point of parturition, the mammary gland, once comprised of mostly adipocytes, becomes an epithelial cell-rich secretory organ capable of producing copious amounts of milk to support the rapid growth rates of the nursing neonate(s). Depending on the varying lengths of lactation time in species, this stage of mammary gland maturity will continue to provide complete nourishment to the nursing youth until they reach an age at which they can hunt or forage for food. Tight control over the cell-cycle must be maintained during this stage, as the secretory cells must produce many milk lipids and proteins but not enter mitosis.

While its hyper-activity has been linked to the progression of cancerous transformation in cells (12, 15), Cdc42 has also been shown to affect normal mammary gland developmental functions as well (141, 142). This suggests that the general inhibition of Cdc42 may not be an ideal therapeutic approach to breast cancer, but rather the targeting of select Cdc42 signaling could

prove to be beneficial. To better understand the roles of Cdc42 in mammary epithelial cells, loss-of-function as well as overexpression studies have been performed in the mouse during the morphogenesis of the gland. Primary mouse mammary epithelial cells (MECs) showed stunted proliferation rates and reduced survival when cultured in the absence of Cdc42 expression (142). Cdc42-deficient MECs also failed to form organized alveolar acini, due to deficiencies in establishing apical-basal cell polarization and improper mitotic spindle orientation during division. Surprisingly, the defects in proliferation and survival were only seen when primary MECs were grown in a three-dimensional substratum, and not in monolayer conditions. This key point further underscores the impact of the extracellular environment on Cdc42-mediated cell signaling during tissue development, function and maintenance.

Conversely, the overexpression of Cdc42 in the developing mammary gland results in hyper-budding and trifurcation in the terminal end bud units, with increased ductal side-branching (143). These alterations in the development of the mammary gland were not caused by changes in cell proliferation or survival, but rather by an increase in cell migration via mitogen-activated protein kinase (MAPK) signaling. These phenotypes were also accompanied by changes in the stromal environment of the epithelial bed, illustrating the nature of the functional dependence among cell types within the mammary gland. When combining the results from these studies with those examining Cdc42 function in other tissues of the mouse, Cdc42 signaling can be described as essential for not only the function of an individual cell, but also for the concerted efforts of cells that work to together at the tissue-level.

Summary

While the studies described above have shown significant importance for proper Cdc42 signaling during early stages of development in the mammary gland, much less is known about

the potential roles of Cdc42 during the maintenance of function of the adult-stage mammary gland. As breast cancer is a disease primarily afflicting adult women, I began the work presented in this dissertation by investigating the potential roles of Cdc42 in the adult-stage mouse mammary gland. I performed this by using a loss-of-function approach in adult mouse mammary glands, with the goal of identifying the cellular and tissue changes that accompany this loss of Cdc42 expression.

The conditional deletion of Cdc42 in adult lactating mammary glands resulted in abnormal cell-to-cell communication, due to a loss of proper adherens junctions and apical-basal cell polarity. The loss of proper adherens junctions between the mammary epithelial cells was caused by changes in E-cadherin localization and expression, and I reasoned this to be related to aberrant IQGAP1 activity in the absence of Cdc42. The loss of apical-basal cell polarity was caused by a loss of Par6 localization at the apical surface of the mammary epithelial cells, accompanied by aPKCζ mislocalization. Overall, this led to a disorganized architecture within the epithelial compartment of the adult mammary gland, and the cells that lost Cdc42 expression began to prematurely slough off into the central lumens of the alveoli. This phenotype led to stunted lactation potential in the female mice, and demonstrated the essential roles for Cdc42 in the adult mammary gland. This model system allowed us to visualize which of the many potential roles of Cdc42 were relevant to adult-stage mammary gland function. I then wanted to use this information to further probe the effects exerted by Cdc42 during breast cancer progression, and possibly identify how the roles for Cdc42 shift during the process of transformation.

To accomplish this, I employed a primary cell culture model system that allowed for the isolation of mammary epithelial cells from mice which would then be cultured in either monolayer or three-dimensional (3D) conditions. The deletion of Cdc42 in the 3D model system resulted in a phenotype that corroborated with our lack-of-function mouse model, in that disorganized

mammary alveoli formed, consisting of epithelial cells that abnormally occupied the central lumen space. However, the addition of the hyper-active Cdc42[F28L] mutant to the cells in this model system showed an intriguingly different result. While the lack of Cdc42 in MECs, both in the three-dimensional cell culture and mouse systems, resulted in significant changes in cell-to-cell communication and organization, the addition of the hyper-active mutant of Cdc42 resulted in disorganized mammary epithelia that showed invasive characteristics. The invasive nature of the MECs that expressed Cdc42[F28L] was dependent on Cdc42 signaling to IQGAP1, highlighting this effector as a major player in Cdc42 signaling in the mammary gland. Cdc42[F28L] expression in the primary MECs resulted in invasive projections extending out into the laminin-rich extracellular matrix. These invadopodia progressed into the surrounding extracellular matrix, leading to further cellular migration and invasion away from the primary site of cell growth. The phenotypes attributed to the primary MECs in the presence of Cdc42[F28L] are of relevance to cancer biology, as not only are the cells disorganized, but they are invading out into their surroundings.

As the stages of breast cancer advance from ductal carcinoma in-situ to invasive ductal carcinoma, cancer cells within the tumor gain the ability to invade surrounding tissue, potentially reaching the lymphatic or blood flow and metastasizing. As Cdc42[F28L] expression caused normal, differentiated MECs to adopt an invasive phenotype, the role of Cdc42 hyperactivity during this process will be further examined. In particular, I am interested in knowing whether there is a hijacking by Cdc42[F28L] of the role of IQGAP in bundling microtubules at the plasma membrane of cells to aid migration and invasion, as well as in increasing the degradation of E-Cadherin via increased Src activity. These data place Cdc42 as a type of biosensor in mammary epithelial cells, where variances in the levels of its activity and localization leads to selective

binding to effectors that can promote shifts from static, strongly-adhered cells into motile, weak-adhesion cells and back again.

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Chapter 2

An Essential Role for Cdc42 in the Functioning of the Adult Mammary Gland

Abstract

The Rho family small GTPase Cdc42 has been implicated in a wide range of cellular functions including the establishment of cell polarity and the remodeling of the actin cytoskeletal architecture, resulting in the tight regulation of cell growth and survival during developmental processes. The complete knock-out of Cdc42 in the mouse is embryonic-lethal, and its targeted deletion in various tissues has been shown to disrupt tissue homeostasis. Thus far, in most studies, the targeted deletion of Cdc42 occurred during embryogenesis. Here, we have used a conditional gene deletion strategy in mice to probe the specific role of Cdc42 during adult mammary gland function. Cdc42 conditional-knock-out females were unable to adequately nourish their pups, due to a disorganized epithelial compartment within their mammary glands. A closer examination showed that their mammary epithelial cells were not able to maintain functional alveolar lumens, due to an inability to establish normal apical/basal epithelial polarity, as well as proper cell-cell contacts. Loss of these essential epithelial characteristics led to a premature sloughing off of the Cdc42-null epithelial cells. Overall our findings demonstrate that Cdc42 plays essential roles in mammary gland function post pregnancy, where it helps to establish proper epithelial cell polarity and tissue homeostasis during lactation.

-The research described in Chapter 2 is from Druso, JE et al. (2016). J. Biol. Chem. ***291****, 8886-8895*

Introduction

The Rho family GTPase Cdc42 has been shown to function as a key regulator in establishing and maintaining epithelial structures in a broad spectrum of multi-cellular organisms, ranging from *Caenorhabditis elegans* to *Homo sapiens* (1-3). *In vitro* two-dimensional (2D) and three-dimensional (3D) cell culture systems have provided molecular insights into how Cdc42 regulates the establishment and maintenance of epithelial cell polarity and morphology. In 2D cultures of Madin-Darby canine kidney (MDCK) cells, Cdc42 was shown to be involved in the maintenance of epithelial cell morphology, by regulating polarized membrane transport, cell-cell adhesion, and cytoskeletal remodeling (4). Studies using a 3D cell culture system comprised of MDCK cells suggested that Cdc42 is necessary for proper apical membrane specification (5,6), while an analogous system using colorectal carcinoma Caco-2 cells showed that Cdc42 regulates directional vesicular trafficking and mitotic spindle orientation, but not apical-basal cell polarity during cyst formation (7). Together, these findings demonstrate that Cdc42 regulates epithelial cell morphology at multiple levels, and that its specific functional roles are dependent on the cellular context.

Those actions of Cdc42 that ensure the proper maintenance of epithelial structures served as a forecast of critically important functions for this GTPase in various developmental processes. Indeed, a number of studies using conditional knock-out (CKO) mice have shown that the deletion of Cdc42 in epithelial stem/progenitor cells from a variety of tissues results in the disruption of intact epithelial structures, leading to severe and even lethal defects in embryonic organogenesis and tissue homeostasis (8,9). Defects in epithelial structure that accompany the deletion of Cdc42 have been suggested to affect cell fate determination, proliferation, survival, and differentiation during embryonic development (10-14).

The importance of Cdc42 in the development of the mammary gland, prior to pregnancy, has been suggested from studies using transgenic mice, as well as from experiments performed with primary epithelial cells derived from conditional knock-out mice. The virgin mouse mammary gland contains sparse ductal networks that extend into the mammary fat pad and culminate in terminal end bud units. Upon pregnancy, the mammary gland becomes a high-output secretory tissue, largely through the signaling cues of prolactin and progesterone, which instruct the virgin epithelial bed to undergo rapid proliferation to fill the entire mammary fat pad with alveoli that are capable of milk secretion (15). At the time of parturition, luminal epithelial differentiation and lactogenesis begin within the alveoli, and continue through the early stages of newborn life, providing sufficient nutrients to sustain the significant and rapid growth rates of the suckling neonates. After weaning, the expanded milk-producing alveoli undergo massive-scale apoptosis, termed involution, with the entire mammary gland regressing back to a pre-pregnancy-like state, awaiting the next round of pregnancy and lactation (15).

The inducible expression of Cdc42 in pre-pregnant mammary ductal epithelial cells caused hyper-branching of mammary ducts and deformation of terminal end bud units (16). A modest increase in Cdc42 expression (1.5 fold) was sufficient for pre-pregnant mammary ductal epithelial cells to exhibit more invasive phenotypes, thus suggesting that the tight regulation of Cdc42 function is essential for this stage of mammary gland development (16). Studies in 3D cell culture model systems, using pre-pregnant stage primary mammary epithelial cells, showed that the deletion of Cdc42 inhibited acinar formation by causing defects in apical-basal cell polarity, cell-cell contact, mitotic spindle orientation, cell proliferation, and cell survival (17). Additionally, the expression of the dominant negative Cdc42(T17N) mutant also inhibited the establishment of acinar structures and, consequently, prolactin-dependent synthesis of milk

proteins in mammary epithelial cells (18). However, introducing the Cdc42(T17N) mutant into established acini did not affect prolactin-induced milk production, suggesting that Cdc42 is essential for establishing acinar structures but not for milk production in mammary acini (18).

While the studies described above show that Cdc42 plays an important part in normal mammary development before pregnancy, thus far, it has not been demonstrated whether Cdc42 exerts additional *in vivo* functions during the entire process of lactation. Therefore, in order to investigate the importance of Cdc42 in mammary alveolar epithelial cells during lactation, we generated conditional knock-out mice in which Cdc42 is deleted specifically in milk-producing alveolar epithelial cells. This was achieved by crossing floxed Cdc42 (*Cdc42*$^{flox/flox}$) mice with transgenic mice expressing Cre recombinase under the control of the whey acidic protein (WAP) promoter (19). We found that the deletion of Cdc42 in the lactating alveolar cells of female mice prevented them from being able to sufficiently nourish their pups. This was due to a severe impairment of mammary alveoli formation, resulting from the loss of intact luminal epithelial structures and premature cell sloughing. Interestingly, alveolar cells in Cdc42 conditional knock-out (CCKO) lactating mammary glands still maintained their function as milk-producing cells, and did not exhibit significant defects in cell proliferation and survival during lactogenesis, nor did they show any sign of cellular apoptosis even within the time window when control glands underwent the early stages of involution. When taken together, our current findings show that the ability of Cdc42 to establish cell polarity and cell-cell contacts plays an essential role in ensuring the proper homeostasis of lactating mammary glands. Moreover, they demonstrate that Cdc42 is a key regulator of mammary gland development and function, not only during the pre-pregnancy developmental stage, but also during the post-pregnancy period of lactogenesis.

EXPERIMENTAL PROCEDURES

Mouse strains and husbandry- Mice used in this study were housed and handled in accordance with the Cornell University Institutional Animal Care and Use Committee (IACUC). *Cdc42*$^{flox/flox}$ mice (controls) were crossed to *WAP-Cre(+) Cdc42*$^{wt/flox}$ mice to generate conditional deletion females genotyped as *Cdc42*$^{flox/flox}$*;WAP-Cre(+)*. These females were then mated at the age of eight weeks, and removed from their breeding partner male after a mating plug was observed. For fostering experiments, litters were fostered to a timed mating-matched mother on day one post-partum.

For pup growth experiments, litter size was normalized to 6 pups by fostering, and then each pup was tail marked for identification. The mass of each pup was recorded daily until the age of 21 days, at which point they were weaned from their mother and placed on standard rodent chow. Pup growth measurement was repeated for five litters, resulting in the measurement of 30 pups total for each strain of mouse.

For tissue collection, mice were euthanized by carbon dioxide inhalation, and then the fourth pair of mammary glands was harvested.

Antibodies- Antibodies used for Western blotting and immunohistochemistry or immunofluorescence were purchased from the following companies: anti-Cdc42 (ab64533), anti-Ki67 (ab15580), anti-aPKCζ (ab4139), anti-N-Cadherin (ab12221), anti-Twist (ab50581), anti-Cytokeratin 8 (ab59400), anti-TGFβ1 (ab92486), and anti-Cytokeratin 14 (ab53115) antibodies were from Abcam Inc; anti-phospho-Stat 5 (CS9359), anti-Snail (CS3879), anti-vinculin (CS 4650), anti-phospho-SMAD 2/3 (CS8828) and anti-E-Cadherin (CS3195) antibodies were from Cell Signaling Technology. Anti-cleaved caspase-3 (AB3623) antibody was purchased from

EMD Millipore, anti-β-actin (MA5-15739) was purchased from ThermoFisher Scientific, and anti-Par 6 (25525) antibody was obtained from Santa Cruz Biotechnology.

SDS-PAGE and Western blot analyses- Immediately following euthanasia, the fourth pair of mammary glands was harvested from mice, frozen in liquid nitrogen, and ground using a mortar and pestle. The tissue powder was then suspended in triple detergent buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide, 1 mM sodium vanadate, pH 8.0) with protease inhibitors (10 µg/mL leupeptin, 10 µg/mL aprotinin) and incubated on ice for 20 minutes. The extracted proteins were centrifuged at 13,000 rpm for 15 minutes at 4° C, after which the supernatant was removed and used for BioRad protein assays. Samples were subjected to polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Perkin Elmer). The membranes were blocked using 5% milk, and incubated overnight on a rocker at 4° C with the primary antibodies at a dilution of 1:1000. Membranes were then washed three times for five minutes in TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 1 hour, after which an ECL reagent (Perkin Elmer) was used to obtain chemiluminescence of the signal onto x-ray film.

Whole mount staining- Whole mount staining of the entire mammary gland was performed by spreading the gland onto a Superfrost Plus slide (VWR) and then placing another slide on top. These two slides were compressed together for 15 minutes, with the mammary gland between them, using two 2-inch binder clips. The top slide was then removed and the mammary glands were fixed in Carnoy's fixative (6 parts 100% ethanol, 3 parts chloroform, 1 part glacial acetic acid) for 6 hours, after which they were rehydrated using a succession of 20-minute ethanol washes (70%, 50%, 25%), and finally, distilled water. Epithelial staining was

achieved by submerging the mammary glands (still mounted on one slide) in carmine aluminum staining solution, containing 0.2% carmine aluminum, 0.5% aluminum potassium sulfate, and a thymol crystal, overnight at room temperature. After staining, the tissues were dehydrated by performing 20-minute ethanol washes (70%, 95%, 100%), followed by clearing of the tissue through two 20-minute washes in mixed xylenes, and mounted and coverslipped using Permount. Imaging was performed using an Olympus SZ-11-CTV dissecting microscope fitted with an Olympus Camedia C-5050 digital camera.

Immunohistochemistry and immunofluorescence- The fourth pair of mammary glands were harvested from pregnant or lactating females, rinsed briefly in PBS, and fixed in 4% paraformaldehyde for 15 hours at 4° C. These glands were embedded in paraffin, sectioned at 5 µm thickness, and placed onto VWR Superfrost slides.

For immunohistochemistry staining, the tissue sections were deparaffinized and rehydrated, and subjected to heat-induced epitope retrieval (HIER) using either an EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) or a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Antigen retrieval buffers were heated to 95°C, at which point sample slides were added, and then kept at 95° C for 15 minutes to induce antigen retrieval. Slides were left in the heated antigen retrieval buffer to cool at room temperature for 15 minutes, after which they were rinsed using three 5-minute PBS washes. Samples were processed using an ImmunoCruz ABC staining kit (Santa Cruz) along with the primary antibodies, dehydrated by 5-minute washes with varying amounts of ethanol (70%, 95%, 100%), cleared by two 5-minute washes of mixed xylenes, and mounted with Permount (Fisher Scientific). Imaging was performed using an Olympus AX70 compound microscope equipped with a MicroFire camera and PictureFrame image processor (Optronics).

For immunofluorescence staining, tissue sections were deparaffinized, rehydrated, and subjected to HIER as above, and then blocked in 10% bovine serum albumin in PBS for one hour at room temperature in a moisture chamber. Primary antibody dilutions were added to the samples, which were replaced in the moisture chamber and incubated for 15 hours at 4° C. Samples were then removed from the primary antibody dilutions, and rinsed in four 5-minute PBS washes, followed by incubation in Alexa Fluor 594-conjugated antibody (Life Technologies) for one hour at room temperature in a moisture chamber. Samples were rinsed in three 5-minute PBS washes, and incubated in 4,6-diaminodino-2-phenylindole (Sigma Aldrich) at a 1:5000 dilution in double distilled water (ddH_2O) for 5 minutes at room temperature. Samples were then rinsed using three 5-minute ddH_2O washes, and sealed with KPL Fluorescent Mounting Media. Epifluorescence imaging was performed on a Zeiss inverted Axioskop equipped with a COOKE Axiocam/Sensicam camera.

For Fast Green/Sirius Red staining, the tissue sections were deparaffinized by two 10-minute washes of mixed xylenes (Fisher Scientific), and rehydrated by successive 5-minute washes with different amounts of ethanol (100%, 95%, 70%), followed by two 5-minute washes with ddH_2O. The tissue sections were then immersed in a solution of 0.1% Fast Green, 0.1% Sirius Red, and 1.2% picric acid for 30 minutes at room temperature, dehydrated by 5-minute washes with 95% and 100% ethanol, cleared with two 5-minute washes of mixed xylenes, and mounted with Permount (Fisher Scientific).

Primary mammary epithelial cell isolation and 3D culture- The fourth pair of mammary glands were immediately harvested from euthanized twelve-week-old virgin *Cdc42*$^{flox/flox}$ female mice. Following the protocol from Lo et al. (20), the glands were minced briefly with two sterile razor blades, then digested at 37° C with 100 rpm rotation in digestion buffer (2 g/l trypsin

(Invitrogen), 2 g/l collagenase type-iv (Invitrogen), 5% v/v FBS (Invitrogen), and 5 µg/ml insulin (Sigma) in DMEM/F12 medium (Invitrogen)). Digested mammary glands were then centrifuged at 400 g for 15 minutes at room temperature. The top 15 ml of fat cell-containing media were pipetted off and resuspended in DMEM/F12, and then centrifuged again at 400 g for 15 minutes at room temperature, along with the original resuspended pellet of organoids. The resulting pellets were then resuspended and combined in DMEM/F12 and centrifuged at 400 g for 15 minutes at room temperature. This pellet was resuspended in DMEM/F12 containing 2 U/µl DNase 1 (Sigma) for 5 minutes at 37 ° C with gentle swirling. The suspension was centrifuged at 400 g for 15 minutes at room temperature, after which the pellet was resuspended in DMEM/F12 and pulse-centrifuged twice at 260 g at room temperature. The pellet was resuspended in DMEM/F12 containing 1x Insulin-Transferrin-Sodium Selenite (ITS, Sigma) and 1x Penicillin/Streptomycin (PS, Invitrogen), and organoids were counted. The solutions were centrifuged at 260 g for 15 minutes at room temperature, after which the pelleted organoids were resuspended in Growth Factor Reduced Matrigel (BD Biosciences), and plated in 8-well chamber slides at approximately 200 organoids per well. DMEM/F12/ITS/PS (300 µl) were added to the top of the matrigel, and the organoids were cultured at 37° C and 5% CO_2 in an incubator overnight. After 24 hours, the media was exchanged with DMEM/F12/ITS/PS containing 9 nM TGFα (Sigma). After 6 days of culture, exchanging the DMEM/F12/ITS/PS/TGFα media every second day, the organoids formed hollowed alveoli. Adenovirus was then added to the media: control cultures were given Ad–CMV-GFP virus (Vector Biolabs) at approximately MOI 80, while experimental cultures were given Ad-Cre-GFP virus (Vector Biolabs) at approximately MOI 80. Cells were imaged on an Olympus CK2 inverted microscope with an Olympus Camedia C-5050 digital camera.

RNA sequencing- The fourth pair of mammary glands was harvested from euthanized mice at lactation day 5, and flash frozen in liquid nitrogen and crushed with a mortar and pestle. The pulverized tissue was then homogenized using a Qiagen RNA Shredder kit, and RNA was immediately extracted using a Qiagen RNeasy kit. The extracted total RNA was then sequenced at the Cornell University Institute of Biotechnology Genomics Facility. RNA sequences were then analyzed using the TopHat Alignment and Cufflinks Assembly software on Illumina's BaseSpace website, and further analyzed using the iPathway Guide.

RESULTS

Cdc42 knock-out mothers are unable to sufficiently nourish pups.

To knock-out Cdc42 in milk-producing alveolar epithelial cells, we mated *Cdc42*$^{flox/flox}$ mice (21) with transgenic mice carrying a Cre recombinase gene under the promoter region for Whey acidic protein (WAP), in which Cre recombinase is expressed specifically in mammary alveolar epithelial cells during lactation (Figure 2.1A). Females that were genotyped either as *Cdc42*$^{flox/flox}$*; WAP-Cre(-)* (control), or as *Cdc42*$^{flox/flox}$*; WAP-Cre(+)* (CCKO), were then mated to induce pregnancy. Western blotting of lysates from the fourth pair of mammary glands showed a reduction in Cdc42 protein levels in CCKO mothers, compared to controls, as lactation progressed (Figure 2.1B).

Pups nursing from CCKO females displayed severely stunted growth rates (Figure 2.1C). To ensure that this phenotype was due to mammary gland defects in the CCKO mothers, and not a result of potential developmental defects in their pups, we fostered pups born to CCKO mothers to control mothers at day 1 post-partum, and vice-versa. Pups born to CCKO mothers had normal growth rates when feeding from a control mother (Figure 2.1D), while pups born to the control mothers showed growth defects when feeding from a CCKO mother. After weaning,

Figure 2.1. The conditional deletion of Cdc42 in the lactating mammary gland stunts nursing pup growth. A) Schematic timeline describing the conditional gene deletion strategy used in this study, with illustrations depicting the development of the mammary gland in each developmental stage. B) Western blot of tissue lysates showing whole-gland Cdc42 expression from the stages of virgin to lactation day 20 (L20), with β-actin serving as a loading control. C) Growth plot depicting average weight of individual pups (n=30) feeding from a CCKO mother (blue), or from a control mother (red), throughout lactation. Error bars represent the standard deviation to the mean pup mass at each given time-point. D) Growth plot depicting average weight of individual pups (n=30) that were fostered from a control mother to a CCKO mother (blue), or from a CCKO mother to a control mother (red). Error bars represent the standard deviation to the mean pup mass at each given time-point.

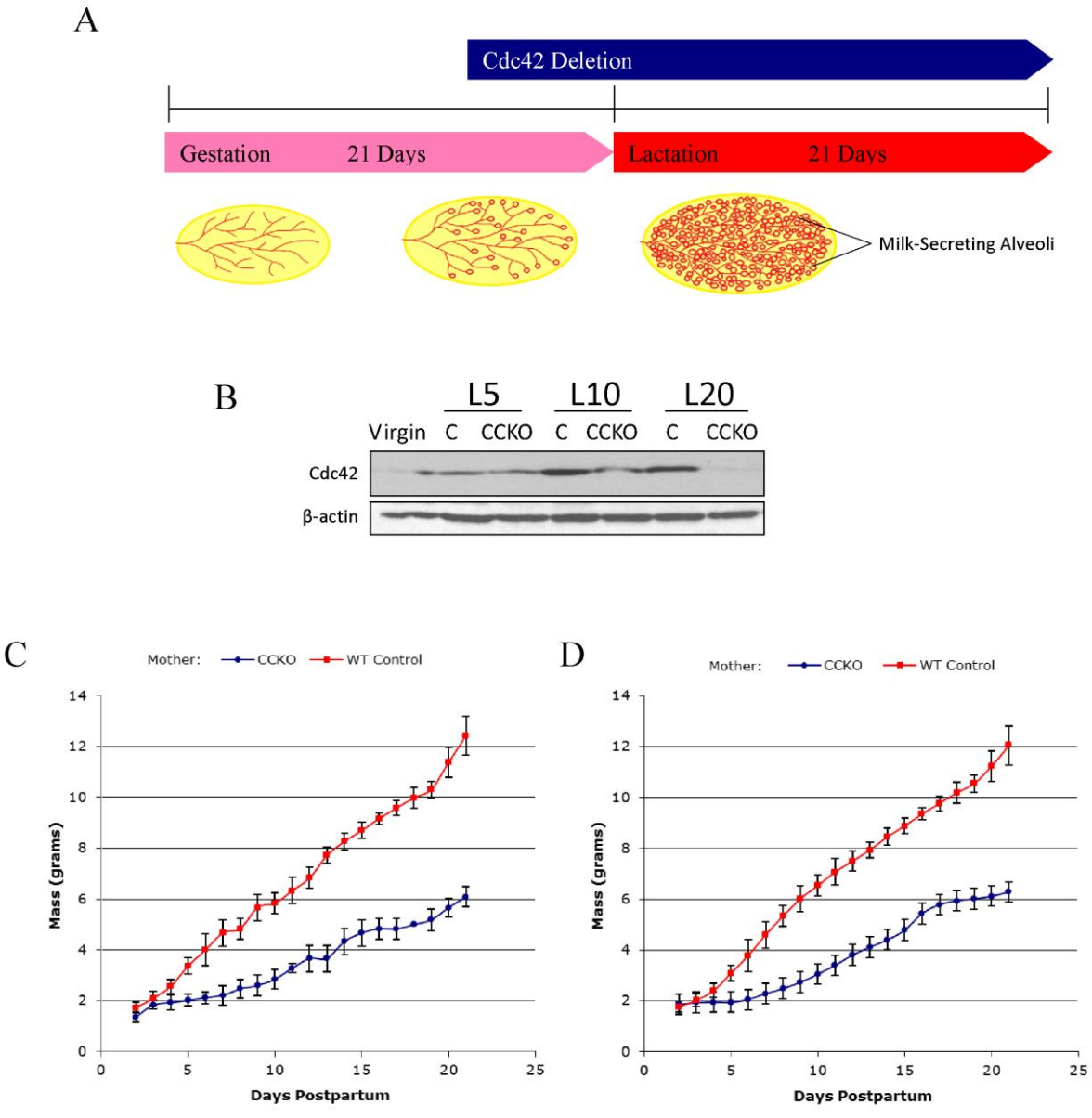
A
Cdc42 Deletion
Gestation
21 Days
Lactation
21 Days
Milk-Secreting Alveoli
B
L5
L10
L20
Virgin
C
CCKO
C
CCKO
C
CCKO
Cdc42
β-actin
C
Mother:
CCKO
WT Control
Mass (grams)
Days Postpartum
D
Mother:
CCKO
WT Control
Mass (grams)
Days Postpartum

Figure 2.1

pups that had nursed from CCKO mothers were able to attain near-normal weights on standard rodent chow, and this growth was accelerated when a milk substitute was added to the food (data not shown). As an added control, pups born to a *Cdc42$^{flox/wt}$; WAP-Cre(+)* mother were analyzed, and followed the same growth rates as pups nursing from the *Cdc42$^{flox/flox}$;WAP-Cre(-)* control mothers (data not shown). Thus, the growth defects exhibited by pups were apparently due to an inhibited secretory output of the CCKO mothers, and not from developmental defects of the pups themselves, nor from Cre expression alone. The pups nursed from CCKO mothers did not exhibit increased mortality, thereby suggesting that CCKO mothers can produce a sufficient amount of milk for pup survival, but not enough to support their normal growth. The same growth defects of pups nursing from CCKO mothers were observed in a second round of lactation of CCKO mothers, after a complete period of involution (data not shown). Given these findings, we set out to perform a more detailed examination of mammary gland development in CCKO mice versus their control counterparts.

Cdc42 knock-out causes an impaired development of lactating mammary glands.

We first examined and compared the overall structures of the mammary glands from CCKO and control females. In whole mount images using carmine red staining, both control and CCKO mothers showed normal ductal epithelial development before pregnancy, and we did not detect any significant difference in ductal elongation, ductal branching, nor in the size and shape of terminal end buds (Figure 2.2, panels *a* and *b*). However, as lactation proceeded, especially at the end of the lactation phase (lactation day 20), the alveolar units of CCKO females appeared significantly underdeveloped, compared to control females (Figure 2.2, panels *c* and *d*). The mammary glands of control mothers showed mature, expanded alveoli, whereas the alveoli of

Figure 2.2. Conditional deletion of Cdc42 in mammary glands inhibits normal alveologenesis during lactation. Whole mount images of mammary glands using Carmine Red staining to visualize the epithelial network in control and CCKO mice in the virgin stage (*a*,*b*), and at lactation day 20 (*c*,*d*). *e*,*f*: Insets from *c*,*d* with arrowheads to indicate fully expanded alveoli in control mice, or latent alveoli in CCKO mice. *g*,*h:* Fast Green and Sirius Red staining depicting the epithelial and collagen basement membrane structures in alveoli sections, respectively. Arrowhead in *h* indicates abnormally sloughing of an epithelial cell. Scale bars represent 50 μm. The results are representative of the analysis of >3 mice each from control and CCKO groups.

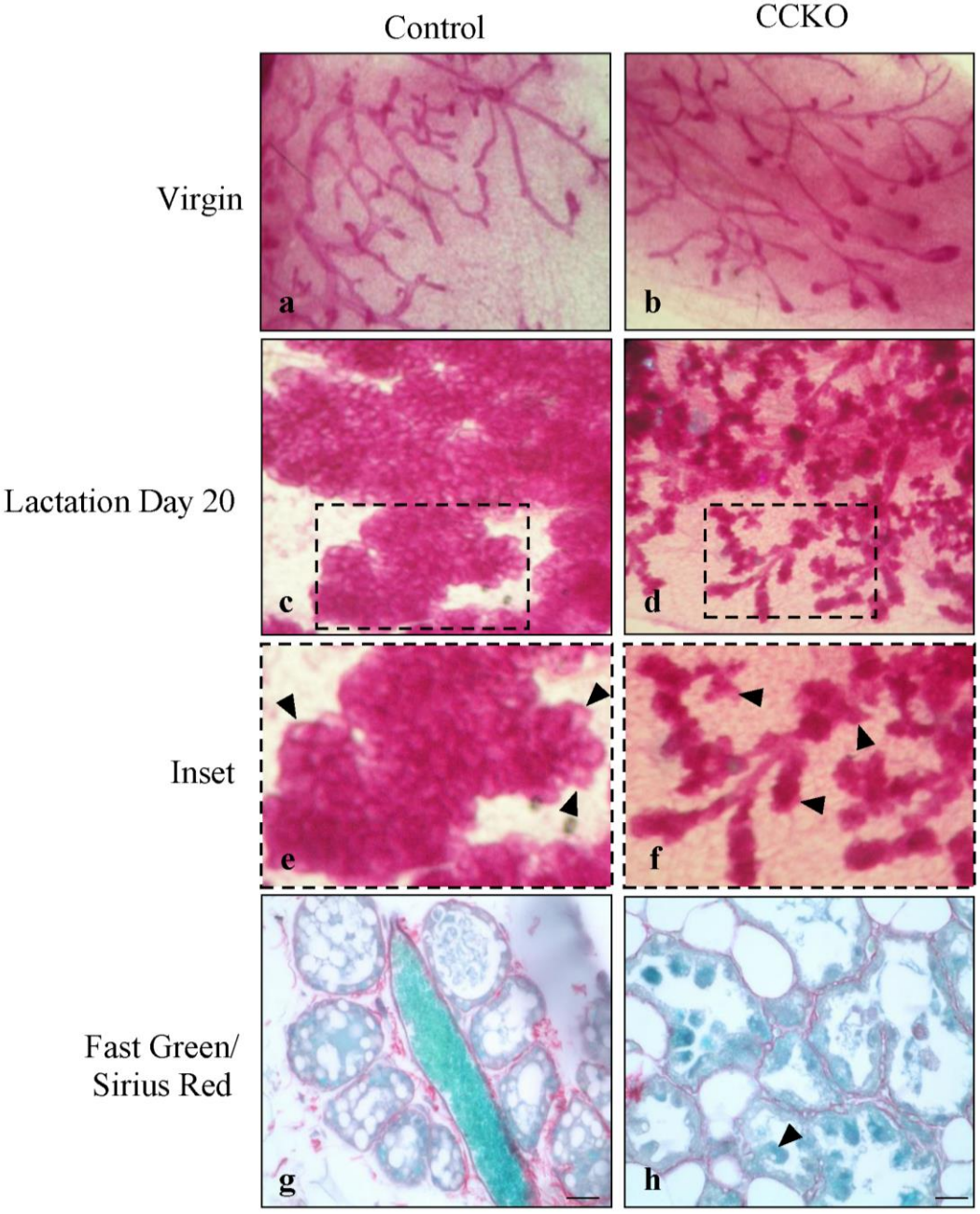
Control
CCKO
Virgin
Lactation Day 20
Inset
Fast Green/
Sirius Red
a
b
c
d
e
f
g
h

Figure 2.2

CCKO mothers showed a significantly latent morphology (Figure 2.2, arrowheads in panels *e* and *f*).

A closer examination showed that the luminal epithelial cells of CCKO mothers exhibited a single-layered structure surrounded by collagen basement membrane, as displayed by staining with Fast Green and Sirius Red, respectively (Figure 2.2, panel *h*). Thus, apparently the deletion of Cdc42 in mammary alveolar cells did not affect the transition of a multi-layered terminal bud unit into a single-layered alveolar epithelium. However, CCKO mammary glands lacked the normal epithelial organization found in control mothers. Alveolar cells of control mammary glands formed a smooth inner luminal surface within each acinus and exhibited a flattened squamous epithelial shape (Figure 2.2, panel *g*). In contrast, many of the inner luminal surfaces of CCKO mammary glands were deformed, and the cells facing alveolar lumens commonly showed cuboidal epithelial structures (Figure 2.2, panel *h*). We also noticed that several cells in the CCKO glands appeared to have sloughed off from the alveolar structures into the inner luminal cavity (Figure 2.2, panel *h*, indicated by arrowhead).

Since the deletion or loss of Cdc42 function has been shown to cause impairments in cell proliferation and increased apoptosis in pre-pregnant mammary ductal epithelial cells (17,18), we examined whether the deletion of Cdc42 in mammary luminal alveolar cells resulted in similar defects, thereby leading to latent alveologenesis. Prolactin-dependent signaling is critical for the proper morphogenesis and secretory capability of mammary gland cells during pregnancy and lactation (15). Activation of the prolactin receptor results in the phosphorylation and activation of STAT5 in lactating mammary glands (22,23). We found that the phosphorylation levels of STAT5 appeared to be maintained and even elevated in CCKO mammary glands in the later stage of lactation (e.g. lactation day 20), compared to controls (Figure 2.3A). These results

Figure 2.3. Changes in proliferation and apoptosis in CCKO mammary glands. A) Western blot of whole-gland tissue lysates depicting phosphorylated Stat5 (pStat5) and cleaved caspase 3 expression throughout lactation in the mammary gland, with β-actin serving as a loading control. L5 = lactation day 5; L10 = lactation day 10; L20 = lactation day 20. B) Immunofluorescence staining depicting Ki67 expression (red) in 5 μm-thick sections from the mammary glands of CCKO and control mice at lactation day 13 (L13, top row) and lactation day 20 (L20, bottom row). Nuclei are stained blue. Scale bars represent 50 μm. The results are representative of the analysis of >3 mice each from control and CCKO groups. C) Quantification of Ki67(+) cells relative to total cells in control and CCKO samples from Fig. 3B. Error bars represent standard deviation to the mean across samples from 3 mice of each genotype group.

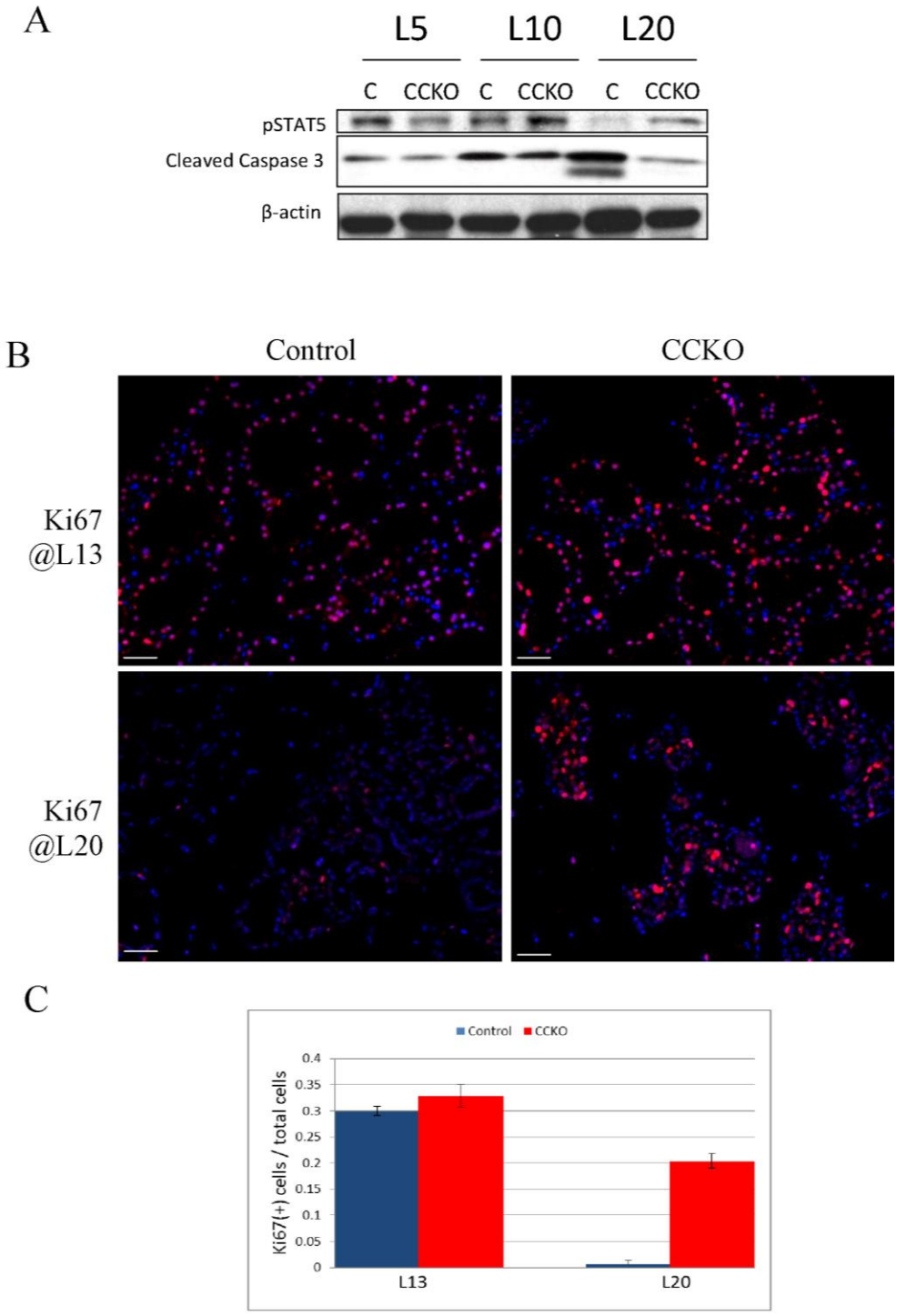
A
L5
L10
L20
C
CCKO
C
CCKO
C
CCKO
pSTAT5
Cleaved Caspase 3
β-actin
B
Control
CCKO
Ki67
@L13
Ki67
@L20
C
Control
CCKO
Ki67(+) cells / total cells
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
L13
L20

Figure 2.3

were corroborated with immunofluorescence staining for Ki67, a marker for mitotic cells, of 5 μm-thick sections excised from control and CCKO mammary glands. At an earlier time point in lactation (lactation day 13), CCKO mammary glands showed only a modest increase in Ki67-positive nuclei, compared to control glands (Figure 2.3B, top two panels). However, at a later time point (lactation day 20), CCKO mammary glands continued to display mitotic activity, while control glands ceased proliferating as they entered into the early stage of involution, such that there was no appreciable Ki67 staining (Figure 2.3B, bottom two panels, and quantification in Figure 2.3C).

We then analyzed the apoptotic activities of control and CCKO mammary glands, using cleaved caspase 3 as a read-out. During lactation (i.e. lactation days 5 and 10), there was little or no difference in cleaved caspase 3 levels, when comparing lysates from control and CCKO mammary glands (Figure 2.3A). However, at the end of lactation (L20), which is the early stage of involution, control glands showed an up-regulation of cleaved caspase 3 levels, whereas CCKO mammary glands did not (Figure 2.3A, third panel from top). Taken together, these results indicate that the underdevelopment of the CCKO mammary glands is neither due to a reduction in cell proliferation nor to an increase in cellular apoptosis.

Deletion of Cdc42 disrupts epithelial cell polarity in lactating mammary alveolar cells.

We next examined whether the defects in mammary gland development exhibited by the CCKO mice were related to alterations in the epithelial structure of the alveoli. At lactation day 13, most alveolar epithelial cells in CCKO mammary glands had much lower levels of Cdc42 expression, compared to control glands (Figure 2.4, red arrows in panels *a* and *b*).

The deformation of epithelial structures in CCKO alveolar tissue (Figure 2.2, panels *g* and *h*) was more evident when cells were immuno-stained with the apical cell surface markers, Par6 and

Figure 2.4. Cdc42 deletion disrupts apical/basal polarity in mammary alveolar epithelial cells. *a,b*: Immunohistochemistry images of 5 μm-thick sections from lactation day 13 depicting Cdc42 expression (brown) in alveolar luminal epithelial cells of the mammary gland from a control mouse (red arrow in *a*), as compared to a CCKO mouse (red arrow in *b*). *c,d*: Immunohistochemistry images depicting the localization of Par6 at lactation day 13. Arrowhead in *d* indicates lipid accumulations. *e,f*: Immunofluorescence images showing the localization of aPKCζ (red) at lactation day 13. Nuclei are stained blue. *g,h*: Immunofluorescence images depicting laminin deposition (red) surrounding an alveolus from control and CCKO mammary glands. Nuclei are stained blue. Dashed lines outline representative alveoli. "L" marks the central lumen of an alveolus. Scale bars represent 50 μm. The results are representative of the analysis of >3 mice each from control and CCKO groups.

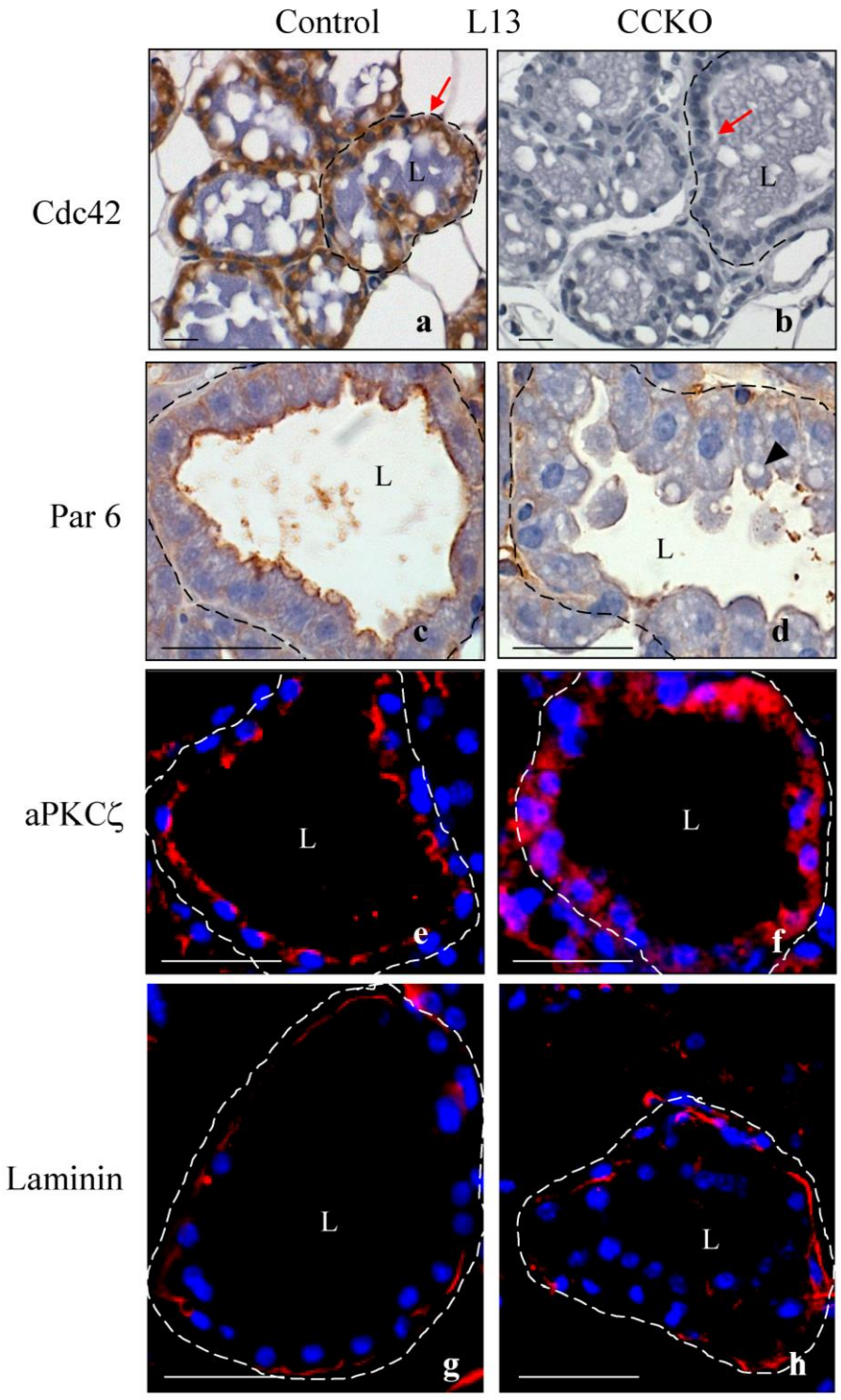

Figure 2.4

atypical PKCζ (aPKCζ). These two proteins form a complex with Cdc42 at the apical plasma membrane, controlling the establishment and maintenance of apical-basal cell polarity 24). In control mammary alveolar tissues, Par6 and aPKCζ were localized at the apical surface of the epithelial cells forming alveolar structures, as shown by immunohistochemistry and immunofluorescence images of 5 μm-thick sections (Figure 2.4, panels *c* and *e*, respectively). However, in CCKO mammary glands, the deformed epithelial cells exhibited only a partial apical membrane localization of Par6, whereas, aPKCζ lost its apical distribution and was diffusely distributed within the cell bodies (Figure 2.4, panels *d* and *f*, respectively).

Since the de-regulation of Cdc42 has been shown to alter the composition of the extracellular matrix, as well as stromal-epithelial cell interactions in pre-pregnant mammary glands (16,25), we examined whether Cdc42 deletion in mammary alveolar cells affected extracellular basement membrane structures. However, both control and CCKO alveolar tissues retained intact laminin deposition (Figure 2.4, panels *g* and *h*), and we did not detect significant alterations in the presence of myoepithelial cells, when comparing cytokeratin 14-immunostaining between control and CCKO mammary alveoli (data not shown).

Deletion of Cdc42 disrupts epithelial cell morphology in lactating mammary alveolar cells.

We next examined cell-cell adhesion structures in control and CCKO lactating mammary alveolar tissues. Immunofluorescence images revealed that control alveolar tissues at lactation day 13 expressed Cdc42 and E-cadherin, and that E-cadherin was localized at basolateral membranes and especially at the cell-cell contacts of epithelial cells (Figure 2.5A, panels *a* and *c*). In CCKO alveolar tissues, Cdc42 deletion was accompanied by E-cadherin being diffusely dispersed within the cell bodies and no longer congregated at cell-cell contacts (Figure 2.5A,

Figure 2.5. Epithelial identity changes in CCKO mammary alveolar epithelial cells. A) Immunofluorescence staining (red) in 5 μm-thick sections from representative control and CCKO mammary alveoli at lactation day 13 depicting the expression and cellular localization of Cdc42 (*a*,*b*), E-cadherin (*c*,*d*), N-cadherin (*e*,*f*), and cytokeratin 8 (*g*,*h*). Nuclei are blue. Dashed lines outline representative alveoli. "L" marks the central lumen of an alveolus. Scale bars represent 50 μm. The results are representative of the analysis of >3 mice each from control and CCKO groups. B) Western blot of whole mammary gland tissue lysates probed for E-cadherin, N-Cadherin, Snail, Twist, TGFβ1, phospho-SMAD 2/3, and cytokeratin 8 throughout lactation, with vinculin serving as a loading control.

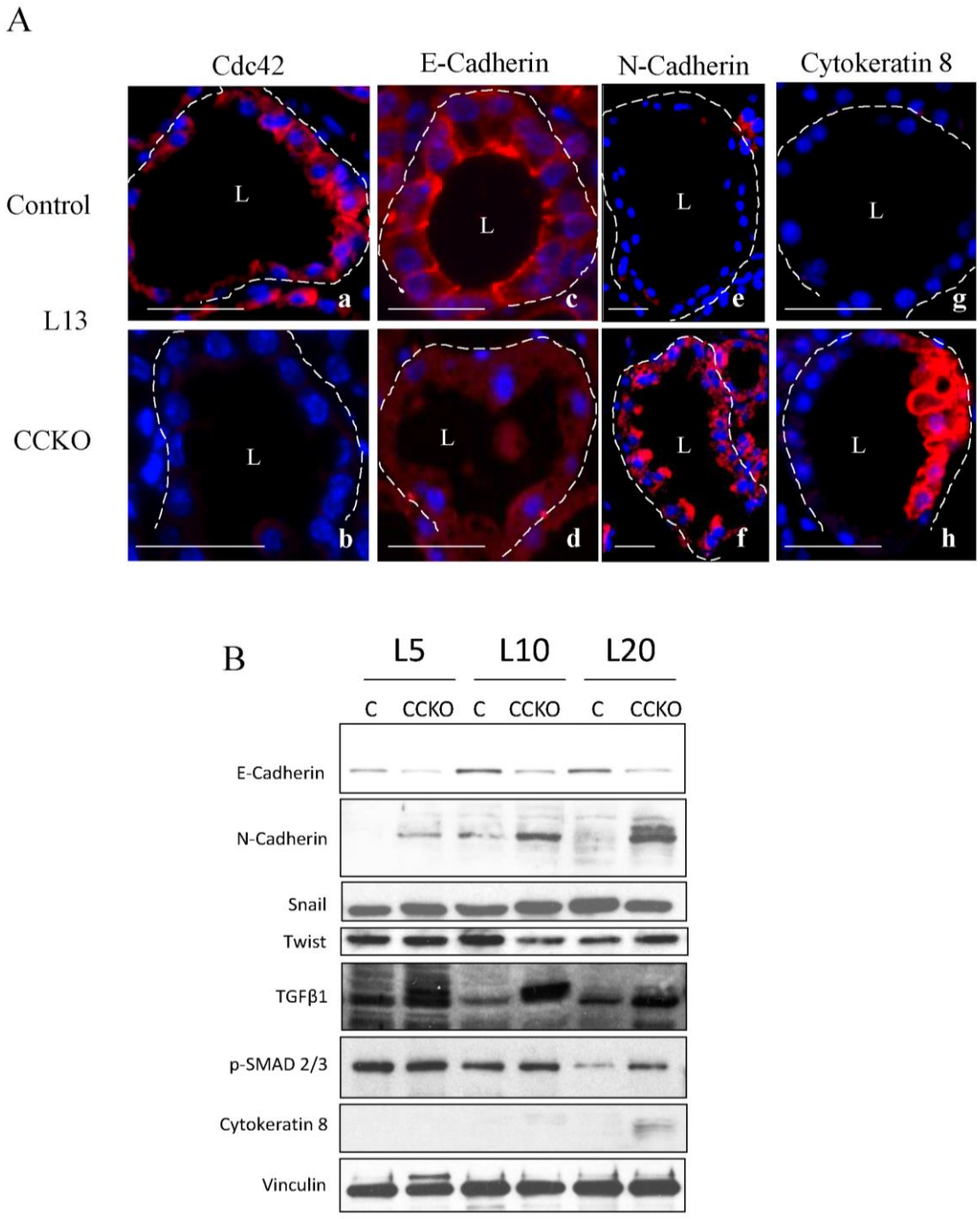
A
Cdc42
E-Cadherin
N-Cadherin
Cytokeratin 8
Control
L13
CCKO
L
a
b
c
d
e
f
g
h
B
L5
L10
L20
C
CCKO
E-Cadherin
N-Cadherin
Snail
Twist
TGFβ1
p-SMAD 2/3
Cytokeratin 8
Vinculin

Figure 2.5

panels *b* and *d*). Collectively, these results suggest that Cdc42 is necessary for the maintenance of cell-cell adhesion as well as apical-basal cell polarity in established mammary epithelial cells, such that its loss leads to disorganized epithelial structures in mammary alveolar glands.

E-cadherin expression was down-regulated in CCKO mammary glands, beginning as early as lactation day 5 and persisting throughout the entire lactation phase, accompanied by an increased expression of N-cadherin (Figure 2.5B). This alternation in the cadherin expression profiles was observed in luminal mammary alveolar cells (Figure 2.5A, panels *c* - *f*). An overall shift in the expression of E-cadherin to N-cadherin often indicates an epithelial to mesenchymal transition (EMT) (26-28). We also found that TGFβ1 signaling through SMAD 2/3 appears to be increased in CCKO glands (Figure 2.5B). However, the expression of Snail, which represses E-Cadherin expression and thereby promotes EMT in several tissues during development (26-28), was not changed in control versus CCKO glands, nor was Twist, another marker of EMT (Figure 2.5B). Thus, these findings suggest that a full EMT has not occurred.

To identify the differentiation status of the cells comprising the CCKO mammary glands, we examined several markers for mammary epithelial cells by immunofluorescence and found that the expression of cytokeratin 8 (CK8) was up-regulated in CCKO alveoli throughout lactation, as compared to controls (Figure 2.5A, panels *g* and *h*, Figure 2.5B). Importantly, although general epithelial structures were disrupted in CCKO glands, lipid accumulation and casein micelles were still apparent in CCKO glands (Figure 2.4, panel *d*, indicated by arrowhead), suggesting that the deformed epithelial cells retained the ability to produce milk.

Deletion of Cdc42 causes premature cell exfoliation in lactating mammary alveoli.

In the preceding sections, we showed that CCKO mammary glands failed to exhibit properly intact epithelial structures, leading to an abnormal alveolar architecture that contained

cells which were detached from the epithelial planes and found within the luminal cavities (Figure 2.2, panel h, indicated by arrowhead). Studies performed using the 3D culture of primary mammary epithelial cells isolated from *Cdc42*$^{flox/flox}$ mice showed that the deletion of Cdc42 (via adenovirus-mediated Cre expression) in antecedently-formed mammary acini caused the epithelial cells to slough off or migrate into the alveolar lumen (indicated by an arrowhead, Figure 2.6). This provided us with a clue regarding the reason for the apparent under-development of the alveoli in CCKO mice (Figure 2.2), and led us to examine whether this phenotype was the result of the mammary alveolar epithelial cells sloughing off into the lumen and being removed by nursing pups. To examine this possibility, we removed the nursing pups from control and CCKO mothers for five hours before harvesting the mammary glands at lactation day 19, thereby allowing any content within the mammary alveolar lumens to accumulate, instead of being removed from the ductal network and ingested by the nursing pups. Upon this treatment, CCKO mammary glands showed an accumulation of Cdc42-negative cells collected within the luminal cavities, while control glands did not (Figure 2.7).

Immunofluorescence staining of serial sections showed that the Cdc42-negative cells, whether floating in the luminal space or still embedded within the alveoli in CCKO mammary glands, were indeed responsible for the up-regulated expression of CK8 (indicated by arrowheads, Figure 2.7, panels *a* - *d*). The floating cells in the CCKO mammary glands did not show cleaved caspase 3-staining (indicated by arrowhead, Figure 2.7 panel *f*), suggesting that these cells were not removed from the epithelial planes due to apoptosis, which we occasionally detected within alveolar lumens in control mammary glands (Figure 2.7, panel *e*). When combined with the findings from the preceding section, these results suggest that CCKO

Figure 2.6. Cdc42 deletion causes luminal filling in primary mammary epithelial cells. Phase contrast images depicting primary mammary epithelial cells forming acini with hollowed lumens at the time of Adenovirus transduction (top panels). Forty-eight hours after transduction, control (Ad-CMV-GFP) acini retain hollowed lumens (bottom left panel), while Cdc42-deletion (Ad-Cre-GFP) acini display cells that have migrated into the lumen (arrowhead, bottom right panel).

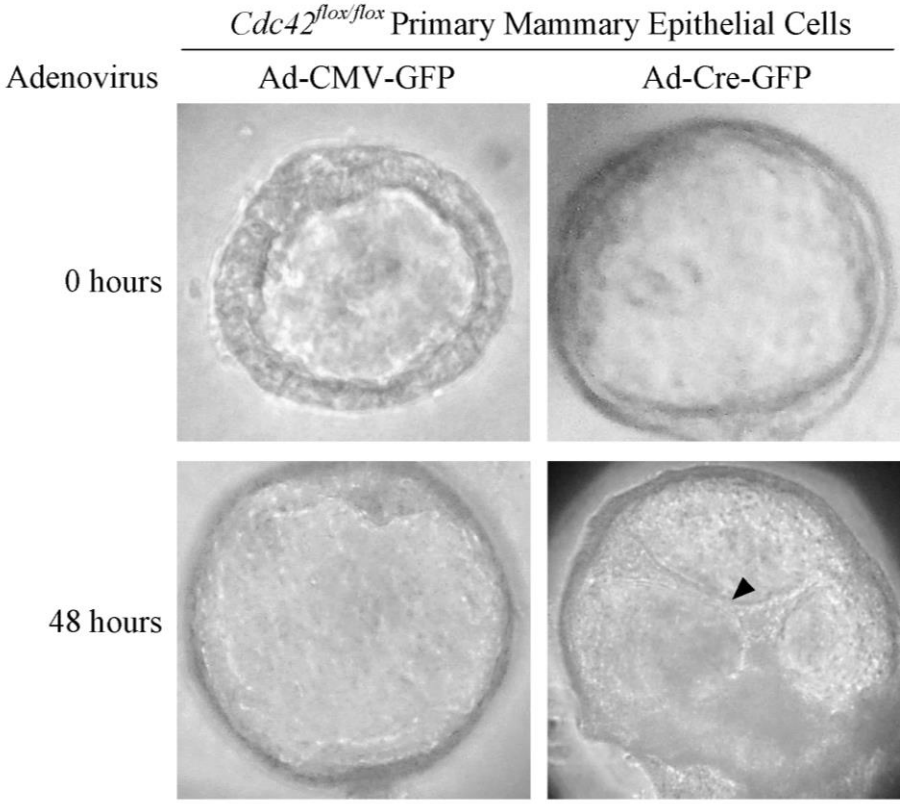
Cdc42flox/flox Primary Mammary Epithelial Cells
Adenovirus
Ad-CMV-GFP
Ad-Cre-GFP
0 hours
48 hours

Figure 2.6

Figure 2.7. Epithelial cell loss in CCKO mammary glands is due to premature sloughing. Immunofluorescence staining (red) in 5 μm-thick sections from representative control and CCKO mammary alveoli at lactation day 13, depicting the expression and cellular localization of Cdc42 (*a*,*b*), cytokeratin 8 (*c*,*d*) and cleaved caspase 3(*e*,*f*). Arrowheads point to prematurely sloughed cells. Nuclei are stained blue. Dashed lines outline representative alveoli. "L" marks the central lumen of an alveolus. Scale bars represent 50 μm. The results are representative of the analysis of >3 mice each from control and CCKO groups.

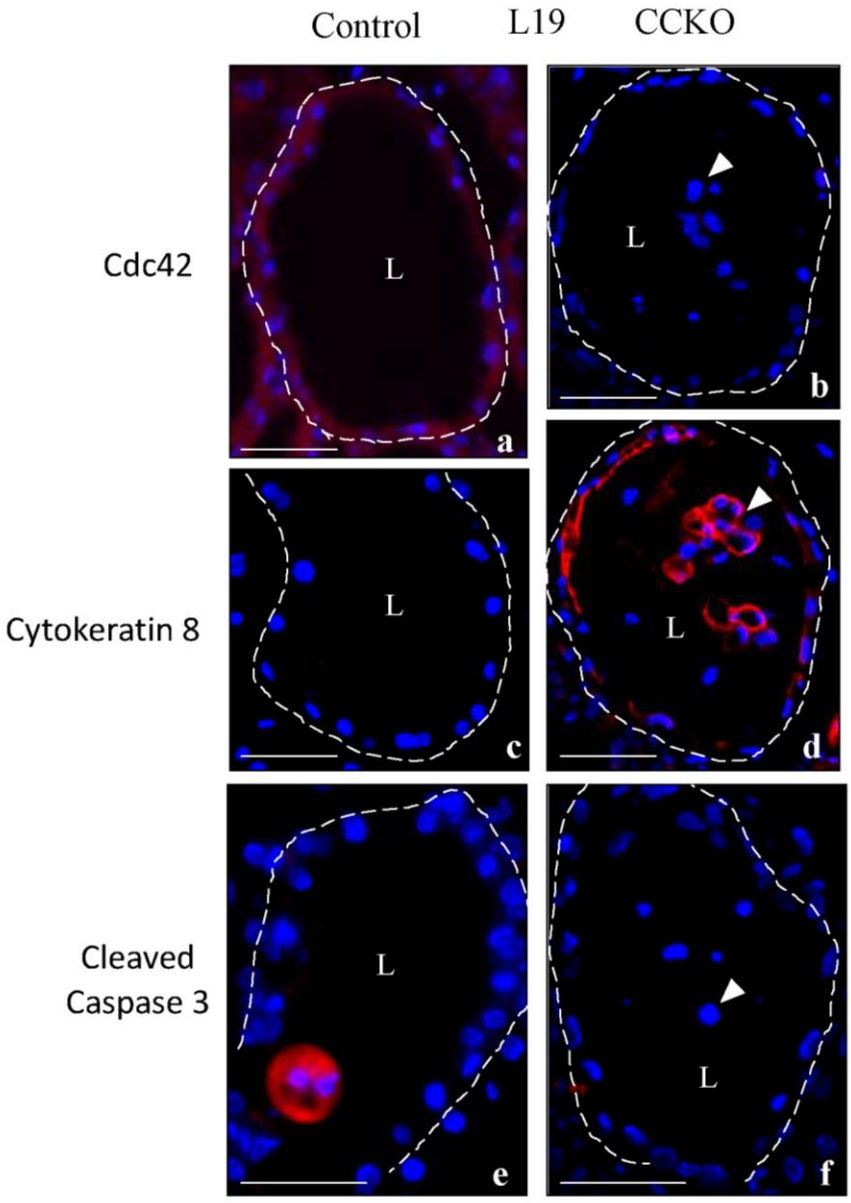
Control
L19
CCKO
Cdc42
Cytokeratin 8
Cleaved
Caspase 3
L
a
b
c
d
e
f

Figure 2.7

mammary alveolar cells lose their communication with neighboring cells, and thereby gain aberrant migratory capability. This results in their detachment from mammary epithelial planes, thereby resulting in an overall impairment in the development and function of CCKO mammary glands.

DISCUSSION

In this study, we have examined the roles played by Cdc42 in adult mammary gland function during lactation. We show that the conditional deletion of Cdc42 results in a marked under-development of mammary alveoli, thus significantly impairing the ability of CCKO mothers to provide the proper nourishment to their pups. This impairment in the development of lactating mammary alveoli is not caused by a reduction in cell proliferation nor through an increase in cellular apoptosis. Rather, our studies show that CCKO mammary alveoli exhibit a latent and under-developed morphology because their alveolar cells prematurely exfoliate from epithelial planes during lactation, which is the result of structural defects and alterations in cell status caused by the deletion of Cdc42.

At least some of the structural defects are likely the outcome of the inability of CCKO mammary alveolar epithelial cells to maintain proper cell polarity after the onset of lactogenesis. Indeed, the deletion of Cdc42 in pre-pregnant mammary epithelial cells has been reported to disrupt intact epithelial structures during acinar formation, due to defects in apical-basal cell polarity, cell-cell adherens structures, and tight junctions (17,18). While Cdc42 has also been suggested to be important for establishing basement membrane structures by controlling stromal-epithelial interactions, as well as for the deposition and processing of extracellular matrix proteins in pre-pregnant mammary glands (16,25), we did not detect any disruption of basement

membrane structures, nor histological differences in stromal layers, in lactating CCKO mammary alveoli.

One of the most obvious changes accompanying Cdc42 deletion in mammary glands during lactogenesis was a marked reduction in E-cadherin expression at the whole gland-level, as well as the disruption of E-cadherin-dependent cell-cell adhesion structures. At earlier lactation periods (lactation day 5), these changes were observed in a subset of the epithelial cell population within the mammary gland as the cells began to lose Cdc42 expression. However, as lactation proceeded (lactation day 13), the diffuse localization of E-Cadherin was more readily apparent throughout the luminal alveolar epithelia. We attribute this to the non-synchronous deletion of Cdc42 throughout the mammary gland at earlier time-points, leading to more thorough gene deletion as lactation proceeded. While the changes observed at the earlier time periods were more subtle than those at later time points, even these subtle differences in the secretory capacity of the mammary gland can have a large effect on the newborn pups' high demand for nutrients (29). These data are consistent with reports implicating Cdc42 in the maintenance of E-cadherin homeostasis through its ability to promote the endocytosis and lysosomal degradation of non-trans-interacting E-cadherin, as mediated by the Par protein complex, WASP, and CIP4 (30,31), and by preventing trans-interacting E-cadherin from being endocytosed, as promoted by IQGAP1 (32,33). Interestingly, the changes in E-cadherin levels observed in CCKO mammary alveoli were accompanied by a significant up-regulation of N-cadherin expression. This led us to examine whether the Cdc42 deletion promoted EMT, whose hallmark phenotype is the shift from the up-regulated expression of E-cadherin to N-cadherin (26-28). However, while we did see elevated TGFβ1 signaling through SMAD 2/3, we did not detect the up-regulation of other marker proteins for EMT, including Snail and Twist (26-28).

Taken together, these data suggest that the deletion of Cdc42 caused a shift in mammary epithelial cell status, but did not induce EMT.

As lactation proceeded, CCKO mammary alveolar cells also exhibited a striking up-regulation of CK8, which has been shown to be expressed in the mammary ductal epithelial cells of virgin female mice, but absent in luminal epithelial cells of the lactating mouse mammary gland (34). Since the alterations in cell status occurred over an extended period of time following the deletion of Cdc42, these changes might be indirect effects caused by Cdc42 deletion. Apparently, the epithelial cell morphology of mammary alveolar cells, as controlled by Cdc42, has an essential role in maintaining their cell identity. Considering that both N-cadherin and CK8 are suggested to be up-regulated in invasive breast cancer (35,36), CCKO mammary alveolar cells, upon losing cell-to-cell contacts and proper tissue architecture, might acquire a shift in epithelial cell identity. This may also explain the ability of the alveolar cells to exfoliate and undergo a premature sloughing off into the lumens.

The lactating CCKO mammary glands exhibited sustained cell proliferation even within the time window when control glands were undergoing the beginning stages of involution (lactation day 20). We also observed a sustained activation of STAT5, which is a key regulator of maturation and cell proliferation in mammary alveologenesis. However, studies using the *in vitro* 3D cell culture model have shown that the introduction of the dominant-negative Cdc42(T17N) mutant into established acini did not affect the prolactin-induced phosphorylation of STAT5 (18). Collectively, these results would suggest that it is unlikely Cdc42 directly regulates prolactin-dependent STAT5 activation. Instead, the loss of Cdc42 may give rise to an overall delay in the steps that control the balance between the cessation of cell proliferation and the onset of programmed cell death necessary for proper adult mammary gland function. Indeed,

we found the levels of activated caspase-3 to be significantly reduced at lactation day 20 in CCKO mammary glands, compared to controls.

Structural deformities in the CCKO mammary tissue might also explain some of the phenotypes accompanying Cdc42 deletion. In the normal development of the mammary gland after weaning, continued milk production increases intra-mammary pressure, which inhibits the secretion of hormones promoting milk production, while inducing the secretion of hormones promoting involution (15). Since CCKO mammary glands are under-developed and lose the cell-cell barriers that normally maintain intra-mammary pressure, CCKO mammary glands might fail to secrete hormones that promote involution.

We also analyzed global gene expression changes between the control and CCKO mice at lactation day 5, by using RNA Seq analysis. Of 23,352 genes and 30,608 transcripts analyzed, only 67 genes and 57 transcripts were found to be differentially expressed with significance between the two samples (data not shown). These differentially-expressed genes mapped mostly to stress response biological programs, as evaluated using iPathway Guide software, which might be an indirect consequence of the abnormal epithelial cell status and premature sloughing of cells in CCKO mammary glands.

In conclusion, our work and that of others suggest that the physiological functions of Cdc42 can be quite diverse and specific to different cell types and stages of development (16-18,25). Here we have shown that the loss of Cdc42 and the inability of CCKO cells to establish proper cell polarity and cell-cell contacts have severe consequences for the functioning of adult mammary glands. Certainly, an important question for the future concerns how the physiological functions of Cdc42 in normal mammary cells correspond to its aberrant behavior in some forms of breast cancer, in which it is over-expressed (37). This could give rise to de-

regulated Cdc42-signaling activities that interfere with the establishment or maintenance of proper cell polarity. Thus, it is easy to imagine how on the one hand, the impact of altered mammary cell polarity and glandular structure that accompanies the loss or de-regulation of Cdc42 can result in improper mammary gland function and lactogenesis, while on the other hand, the over-expression of Cdc42 and its excessive signaling disrupts normal cell polarity and contributes to the transformed state and the malignant phenotypes that accompany breast cancer progression. A better understanding of these Cdc42 functions during the development of different forms of breast cancer could lead to new strategies regarding how and when to target Cdc42-signaling events as possible anti-cancer therapies.

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Chapter 3

The Effects of Constitutively Active Cdc42 on Mammary Alveolar Morphogenesis and Maintenance

Abstract

Cdc42 overexpression and hyperactivity are found in certain human ductal carcinomas of the breast, and a constitutively-active activator of Cdc42, onco-Dbl, confers characteristics of cancer onto normal fibroblast cells, bringing to question the role of this small GTPase during human breast carcinogenesis. The conditional knockout of Cdc42 in the adult mouse mammary gland results in abnormal epithelial organization due to altered cell polarity and cell-cell contacts, traits that are also altered in breast cancer progression. These data suggest a critical role for proper Cdc42 signaling in mammary epithelial cell function and maintenance. Here, we have examined the actions of the hyperactive mutant Cdc42[F28L] in primary mammary epithelial cells (MECs) cultured in both monolayer and 3D conditions. Primary MECs expressing Cdc42[F28L] in monolayer growth caused actin-based stress fiber formation and multi-nucleated cells, while in 3D culture Cdc42[F28L] induced luminal filling and invasive traits in the cells. Further analysis showed that Cdc42[F28L] required binding to IQGAP1 to cause the MECs to acquire invasiveness, but not luminal filling. These findings suggest that aberrant Cdc42 signaling can contribute to multiple aspects of breast cancer progression, including the loss of alveolar luminal architecture and the acquisition of invasive characteristics, and that these traits are due to specific effector binding.

Introduction

Breast cancer research has developed into an enormous field, utilizing an impressive variety of techniques to further our understanding of carcinogenesis. With studies ranging from mammary stem cell biology to cancer cell metabolism and physiological metastasis, our knowledge base continues to grow as we seek more effective breast cancer therapies. Current long-term breast cancer therapy continues to rely on both the ability to prevent further breast tumors from forming after the original tumor is detected, as well as preventing metastasis to other tissues within the patient. However, challenges remain for some breast cancer survivors even after primary tumor excision, as residual cancer cells that are undetected within the breast may still have the potential to form new tumors. Adding to these challenges, the original excised tumor may have already spread to new sites of growth in the patient's tissues before the primary tumor was removed from the patient (1). This latter threat is particularly relevant to advanced, stage IV-type breast cancers and has brought a focus to the study of cancer cell metastasis, with the hope of designing therapies that could prevent breast cancer cells from escaping the mammary gland environment and metastasizing to new tissues (commonly bones, lungs, liver or brain) within the patient.

The likelihood that a cancer cell will metastasize is largely dependent on its ability to migrate and invade (2, 3). Neighboring epithelial and stromal cells, as well as extracellular matrices that have been distributed throughout the tissue, typically help to ensure that cells migrate minimally in adult tissues, and only when temporally required, such as during wound healing (4). Invasive cancer cells are able to overcome the migration-retarding signals provided by the extracellular environment in tissues, in part by displaying traits similar to an epithelial to mesenchymal transition (EMT) (5–7), which is a plasticity developed by epithelial cells that is

critically important during embryogenesis and wound healing. The down-regulation of proteins that confer epithelial cell characteristics (such as E-cadherin), and the up-regulation of proteins that elicit the features of mesenchymal cells (including N-cadherin expression and the release of matrix metalloproteinases) are part of the functional shift required to induce an epithelial cell to acquire the phenotypic properties of a mesenchymal cell. This change in gene expression allows epithelial cells, which are apical-basal polarized and adjoined to neighboring epithelia via adherens junctions, to dissolve these cell-to-cell junctions and establish front-rear polarity, allowing for the mesenchymal cell characteristics necessary for migration. Once the correct extracellular environment is found, the mesenchymal cell can transition back into an epithelial cell by the reverse process, termed a mesenchymal to epithelial transition (MET). This cellular plasticity is critical to the development and function of higher life-forms, yet when acquired by cancer cells, an EMT can turn benign tumor cells into metastatic assassins of their host. For this reason, great efforts have been invested in research toward understanding the shift from an epithelial-like to a mesenchymal-like phenotype, with particular focus on the mesenchymal attributes of invasive migration.

While some of the hallmarks of cancer, such as cell growth in the absence of mitogenic signals and the evasion of programmed cell death, can be studied under monolayer cell culture conditions, the investigation of invasive migration requires a model system that can analyze the ability of cells to invade through an environment composed of multiple cell types and extracellular matrix. To this end, transwell-migration assays have provided a means to study cell migration through an extracellular matrix analog, yet questions of the physiological relevance of this assay still remain (8, 9). Along with this, cells that are grown in monolayer culture conditions are disconnected from their native tissue environmental cues and therefore lose the ability to

recapitulate their physiological control over migration, proliferation, metabolism, differentiation, and death. In response to this challenge, the use of primary epithelial cells cultured in three-dimensional (3D) growth systems has been developed to provide a means of more closely mimicking an *in vivo* environment in which to study complex cellular biology, including processes like invasive migration (10, 11). Studies using mammary epithelial cells (MECs) that are grown in 3D conditions have elegantly illustrated the differences in oncogenic signals that lead either to luminal filling (a condition similar to ductal carcinoma in-situ) or to invasive characteristics that are comparable to invasive ductal carcinoma (12) . As such, this system has provided a valuable method for examining different stages of breast cancer progression, offering researchers a useful bridge between studies performed in monolayer cell culture and those done in an *in vivo* model.

The Rho-family GTPase Cdc42 has been shown to affect multiple cell signaling cascades, resulting in an array of potential downstream cellular effects and biological outcomes (13–15). In reviewing the diversity of effects that can result from altered Cdc42 function, it becomes apparent that the specific Cdc42 signaling outputs that occur are dependent upon the cellular context. This makes it challenging to translate the different Cdc42 functions observed in cell culture models with those that have been identified from studies performed in physiologically-relevant settings, as the varying cell types within our tissues may rely on Cdc42 in different ways. As an example, studies of Cdc42 using fibroblasts grown in monolayer conditions showed that a constitutively-active Cdc42 mutant induced the formation of stress fibers and actin-rich microspikes at the cell periphery (16, 17), but how these functions relate to the embryonic lethality accompanying the deletion of Cdc42 in the mouse (18) is unclear. Therefore, the ability to accurately identify cell signaling pathways that are influenced by Cdc42, while also describing its physiological roles, requires a perspective that can integrate the results from cell culture model systems with those

obtained from *in vivo* studies in animal models. To this end, the culture of primary epithelial cells in both monolayer and 3D culture systems offers the possibility for providing important insights into cell signaling events that occur within a physiological setting.

A study using primary Cdc42 flox/flox MECs that were isolated from the mouse, infected with AdenoCre-virus, and then plated in either monolayer or 3D culture conditions, showed that the functional consequences of Cdc42 expression on MEC function varies between these two conditions (19). Primary MECs grown in monolayer conditions exhibited no detectable changes in cell cycle control in the presence or absence of Cdc42 expression, yet primary MECs lacking Cdc42 grown in 3D culture showed drastically reduced proliferation and survival rates. These findings highlight the complexity of Cdc42 signaling, not only in a cell-type dependent manner but also in the context of the extracellular environment. It was only in the context of the apical-basal polarity that is established within mammary alveoli that Cdc42 was shown to regulate cell-cycle control in primary MECs. As breast cancer cells commonly lose their ability to properly respond to their extracellular environment (20), understanding how normal mammary epithelial cells respond to key signaling proteins like Cdc42 becomes essential to furthering our knowledge of the progression of this disease.

Cdc42[F28L], a constitutively-active mutant, has been shown to confer cancer-like characteristics, such as anchorage-independent growth and growth in low serum, to non-transformed NIH 3T3 fibroblasts in monolayer cell culture conditions (21). As these data were obtained in monolayer growth conditions on immortalized cells, I wanted to determine how Cdc42[F28L] influences primary mammary epithelial cells grown under conditions more similar to their native tissue environment. To address this, I have isolated primary MECs from adult female mice and introduced the expression of Cd42[F28L] during their growth in either monolayer

or 3D conditions. These two growth conditions instruct the epithelia differently, and may also affect how cells interpret the signaling outputs of Cdc42[F28L]. Indeed, I anticipate that by comparing these different cell culture systems, important insights may be gained into the roles of Cdc42 during breast cancer progression.

Experimental Procedures

Primary mammary epithelial cell (MEC) isolation and culture- The fourth pair of mammary glands were harvested from euthanized twelve-week-old virgin *Cdc42*$^{flox/flox}$ female mice. Similar to the procedure described in Chapter Two, and following the protocol from Lo et al (22), the mammary glands were minced for two minutes using two sterile razor blades pressed together, and then digested at 37° C with 100 rpm rotation in digestion buffer (2 g/l trypsin (Invitrogen), 2 g/l collagenase type-iv (Invitrogen), 5% v/v FBS (Invitrogen), and 5 µg/ml insulin (Sigma) in DMEM/F12 medium (Invitrogen)). Digested mammary glands were then centrifuged at 400 g for 15 minutes at room temperature. From this, the top 15 ml of fat cell-containing media was removed and resuspended in DMEM/F12, and then centrifuged again at 400 g for 15 minutes at room temperature, along with the original resuspended pellet of organoids. The resulting pellets were then resuspended and combined in DMEM/F12 and centrifuged at 400 g for 15 minutes at room temperature. This pellet was resuspended in DMEM/F12 containing 2 U/µl DNase 1 (Sigma) for 5 minutes at 37 ° C with gentle swirling. To remove the DNase 1, the suspension was centrifuged at 400 g for 15 minutes at room temperature, after which the pellet was resuspended in DMEM/F12 and pulse-centrifuged twice at 260 g at room temperature to enrich for epithelial cells and remove fibroblasts. The epithelial-rich pellet was resuspended in DMEM/F12 containing 1x Insulin-Transferrin-Sodium Selenite (ITS, Sigma) and 1x Penicillin/Streptomycin (PS, Invitrogen), and organoids were then counted and plated accordingly.

Primary MEC culture and Cdc42 mutant expression- After isolation, Lentivirus containing Cdc42[F28L] or the Cdc42[F28L:F37A] mutant was then added to the organoid suspension at an MOI of approximately 40. These solutions were then spinoculated by centrifugation using 1000g for 20 minutes to enhance viral entry into the cells, after which the pelleted organoids were either resuspended in Growth Factor Reduced Matrigel (Corning) and plated in 8-well chamber slides at approximately 200 organoids per well, or alternatively plated for monolayer growth on 60mm cell culture dishes at a concentration of approximately 200 organoids per dish. After the isolation and suspension of primary organoids in Growth Factor Reduced Matrigel, DMEM/F12/ITS/PS (300 µl) were added to the top of the matrigel, and the organoids were cultured at 37° C and 5% CO_2 in an incubator overnight. After 24 hours, the media was exchanged with DMEM/F12/ITS/PS containing 9 nM TGFα (Sigma). After 6 days of 3D culture, exchanging the DMEM/F12/ITS/PS/TGFα media every second day, control organoids formed hollowed alveoli. For the monolayer growth treatment, organoids were plated on 60mm dishes after harvest and isolation, and supplemented with 1mL of DMEM/F12/ITS/PS. This medium was removed by aspiration after 24 hours, and replaced with DMEM/F12/ITS/PS containing 9 nM TGFα (Sigma). DMEM/F12/ITS/PS medium containing 9 nM TGFα was then exchanged every two days for the remainder of the experiments. For some experiments, Lentivirus was added to the media: control cultures were given an HA-tag virus at an MOI of approximately 80, while experimental cultures were given Cdc42[F28L] or Cdc42[F28L,F37A] virus at an approximate MOI of 80. Cells were imaged on an Olympus CK2 inverted microscope with an Olympus Camedia C-5050 digital camera, with samples presented representing the majority of phenotypes found.

Immunofluorescence staining and imaging- Primary MECs grown in monolayer culture were removed from incubation and all media was aspirated from the 60mm culture dishes. Cells

were then fixed in 4% PFA (dissolved in PBS) for 15 minutes at room temperature. The 4% PFA solution was then aspirated from the 60mm dishes, and the cells were given 3 PBS washes of 5 minutes each, followed by blocking for one hour in 10% Bovine Serum Albumin (BSA)/PBS at room temperature. After removing the blocking solution, primary antibodies were diluted in 10% Bovine Serum Albumin/PBS and added to the cells to incubate overnight at 4° C. Primary antibody dilutions were then aspirated from each dish of cells, and the cells were treated with 5 PBS washes of 5 minutes each, followed by a 30 minute incubation of Secondary AlexaFluor 594-conjugated antibodies (Sigma), which were diluted 1:300 in 10% BSA. Cells were then rinsed again in 5 PBS washes of 5 minutes each, with the third wash containing 4,6-diaminodino-2-phenylindole (Sigma Aldrich) at a 1:10000 dilution. The samples were then coverslipped and sealed using KPL Fluorescent Mounting Media.

Primary MECs grown in 3D culture were removed from incubation, and all media was aspirated from the top of the chambers. The cultures of cells were then fixed by the addition into each chamber of a 1:1 acetone/methanol solution for 30 minutes at room temperature, after which the fixing solution was aspirated. Chamberslides were then disassembled, and primary MECs, while still embedded within Matrigel, were thinly spread across VWR Permafrost glass slides using a p1000 tip and placed on a covered slide warmer for 60 minutes at room temperature. Slides were then placed in 3 PBS washes of 20 minutes each, after which they were immersed in a 10% BSA/PBS blocking solution at 4° C overnight. Slides were then placed into 50mL Coplin jars containing the primary antibodies diluted in PBS. The slides were incubated in primary antibodies overnight at 4° C, after which they were rinsed in 5 PBS washes of 20 minutes each. Secondary AlexaFluor 594-conjugated antibodies (Sigma) were then diluted 1:300 in PBS, and the slides incubated in this solution for one hour at room temperature. Samples were then rinsed at room

temperature in 3 PBS washes of 20 minutes each, and then counterstained with 4,6-diaminodino-2-phenylindole (Sigma Aldrich) at a 1:10000 dilution in double distilled water (ddH_2O) for 10 minutes. Samples were then rinsed in 3 PBS washes of 20 minutes each, and then coverslipped and sealed using KPL Fluorescent Mounting Media.

Epifluorescence imaging for both the monolayer and 3D culture conditions was performed on a Zeiss inverted Axioskop equipped with a COOKE Axiocam/Sensicam camera.

Antibodies- Antibodies used for immunofluorescence were purchased from the following companies: anti-IQGAP1 (ab133490) and anti-Ki67 (ab15580) antibodies were obtained from Abcam Inc; E-Cadherin (CS3195) and anti-pan-actin (CS8456) antibodies were from Cell Signaling Technology; anti-laminin antibody (AB1953) was from Chemicon International (now EMD Millipore).

Results

Comparing the effects of a constitutively active Cdc42 mutant in primary mouse mammary epithelial cells (MECs) cultured in monolayer and three-dimensional systems.

In Chapter 2, Cdc42 was shown to play critical roles in the maintenance of apical-basal cell polarity and cell-cell sensing in the adult mouse mammary gland. These results raised new questions about how the roles in normal adult mammary tissue relate to the actions of Cdc42 in breast cancer. To begin to address these questions, I used the method of primary MEC isolation and culture described in Chapter 2 to obtain Cdc42 flox/flox MECs. However, instead of analyzing the roles played by Cdc42 in the function of MECs and their morphogenesis into alveoli using a loss-of-function approach, in this study I examined the effects of introducing into primary MECs a Cdc42[F28L] mutant, which our laboratory has previously shown to be capable of constitutive GDP-GTP exchange (termed a “fast-cycling” mutant) and of conferring cancer-like growth

characteristics upon NIH 3T3 fibroblasts (21). The use of a monolayer culture system made it possible to examine the effects of Cdc42[F28L] on cell migration, focal adhesion formation and cell division, whereas the 3D culture system enabled us to examine the effects of fast-cycling Cdc42 on mammary alveolar morphogenesis processes. These 3D alveolar morphogenesis processes include cell migration and division, but also encompass cell differentiation and apical-basal polarity, as well as growth inhibition even in the presence of growth factor stimulation. The comparison of the effects of Cdc42[F28L] in these two model systems can highlight the unique roles played by Cdc42 in cell signaling within the context of the extracellular environment, which is highly relevant and carries a great deal of interest in the study of breast cancer progression.

To perform these experiments, organoids rich with primary MECs were isolated from mouse mammary glands and then either plated on cell culture dishes for monolayer growth, or embedded in growth factor-reduced Matrigel for 3D growth. These cultures were then supplemented with TGFα to promote EGFR activity and mitogenic signaling pathways within the MECs (Figure 3.1). Twenty-four hours after plating the organoids in either condition, the MECs appeared similar using phase microscopy (Figure 3.2, compare panels a and b), except that some of the cells plated in the monolayer condition were attaching to the culture dish and migrating away from the plated organoid (red arrowheads in Figure 3.2, panel a). After seven days of TGFα-stimulated growth, MECs growing in monolayer conditions were observed to be dividing and migrating even further onto the culture dish (white arrowheads in Figure 3.2, panel c), while the MECs embedded within the 3D matrix underwent a different type of growth, as they formed hollowed alveolar structures through a process of morphogenesis (Figure 3.2, panel d). The MECs that formed these alveoli in 3D culture displayed an organization similar to that found in mammary

Figure 3.1. Analyzing the Effects of Constitutively-Active Cdc42 Using Monolayer and Three-Dimensional (3D) Primary Cell Culture Systems. Schematic diagram describing the strategy used to culture primary mammary epithelial cells (MECs) in either a monolayer or 3D system. After isolation from mouse mammary glands, MECs plated on culture dishes grow in a monolayer fashion, migrating and dividing in the presence of medium containing TGFα, while MECs embedded in growth-factor-reduced Matrigel undergo morphogenesis into 3D alveoli in the presence of the same medium. Constitutively-active Cdc42 mutants or an empty vector (control) were expressed in the primary MECs growing in either condition using lentivirus infection.

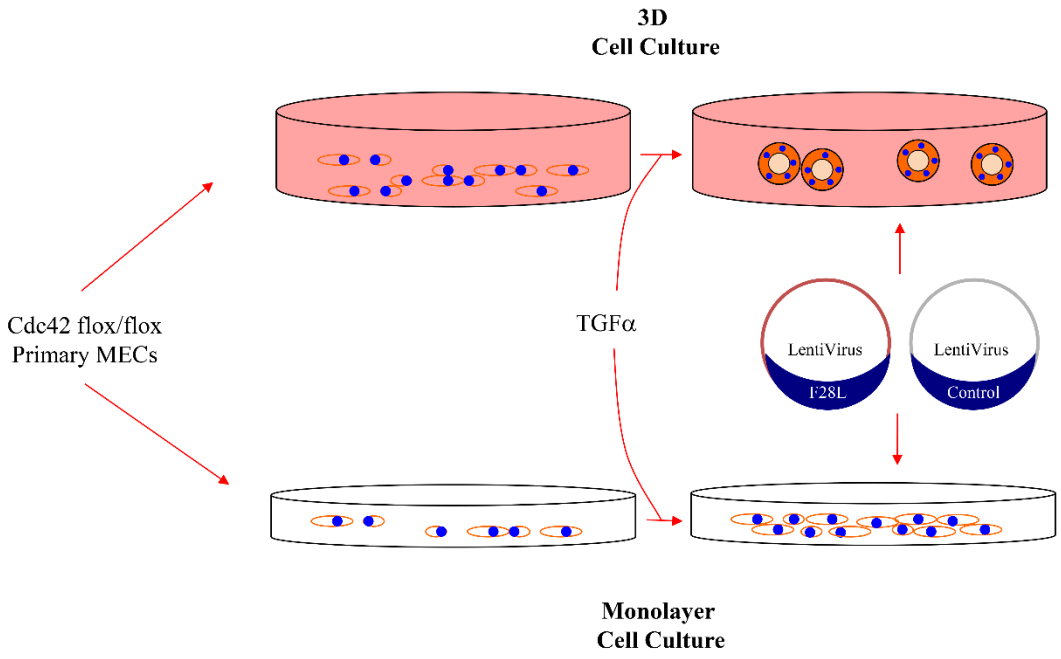

3D
Cell Culture
Cdc42 flox/flox
Primary MECs
TGFα
LentiVirus
I:28L
LentiVirus
Control
Monolayer
Cell Culture

Figure 3.1

Figure 3.2. Primary MECs Display Growth Characteristics Dependent on their Culture Conditions. Phase contrast images depicting primary MEC organoids 24 hours after plating in either monolayer or 3D culture conditions. *a,b:* MECs plated in monolayer conditions are shown attaching to the culture dish and migrating near the bottom of the isolated organoid (red arrowheads in panel *a*), while MECs plated in the 3D culture system appeared as solid organoids within the Matrigel (panel *b*). *c,d:* After 7 days of growth, primary MECs display distinct characteristics in either monolayer or 3D culture conditions. MECs grown in monolayer conditions attached to the culture dish, and displayed flattened morphologies as they migrated onto the substratum (panel *c*). MECs grown in 3D conditions formed hollowed, spherical alveoli, and established an inner lumen (asterisk in panel *d*) lined by the apical surface of polarized cells (red arrowheads in panel *d*). *e,f:* After 7 days of growth, mitotically-active cells were labeled with Ki67 (red) and nuclei were counterstained blue. Many mitotically-active primary MECs were observed in monolayer culture, while few were observed 3D culture (white arrowheads in panels *e,f*).

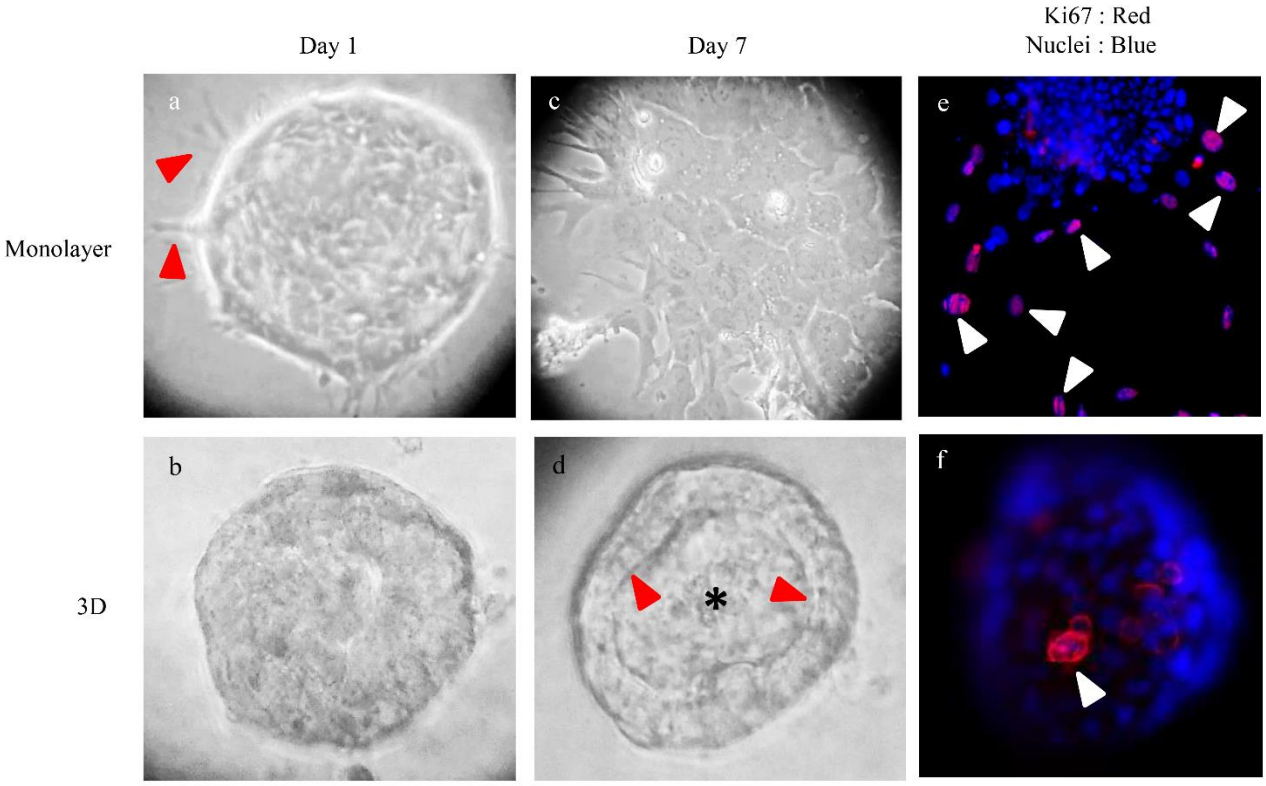


Figure 3.2

gland tissues, where apical-basal cell polarity allows for the formation of an alveolar epithelial cell lining (red arrowheads in Figure 3.2, panel d) that performs a barrier-like function surrounding a hollowed lumenal space (asterisk in Figure 3.2, panel d).

The MECs that were cultured in monolayer conditions and migrating out onto the culture dish became mitotically-active, as indicated by the expression of Ki67 (white arrowheads in Figure 3.2, panel e). However, the MECs cultured in 3D conditions formed alveolar structures that reached a certain size and then largely remained growth-arrested, even when continually supplemented with growth-factor medium (white arrowhead in Figure 3.2, panel f), and survived for at least several months. The MECs grown in 3D culture can be compared to the MECs that comprise the alveolar units in the adult virgin mouse mammary gland, which have undergone some growth to propel morphogenesis, but then remain growth-arrested and "awaiting" the potential hormonal cues of pregnancy (23). The monolayer conditions did not provide the cues to allow the MECs to attain this cell differentiation status and, as such, they continued to migrate and divide until their death, which was within a month after their isolation from the mouse mammary glands. These different cell culture environments represent a window through which to view cell signaling in the presence or absence of morphogenesis-related cues for cellular growth control.

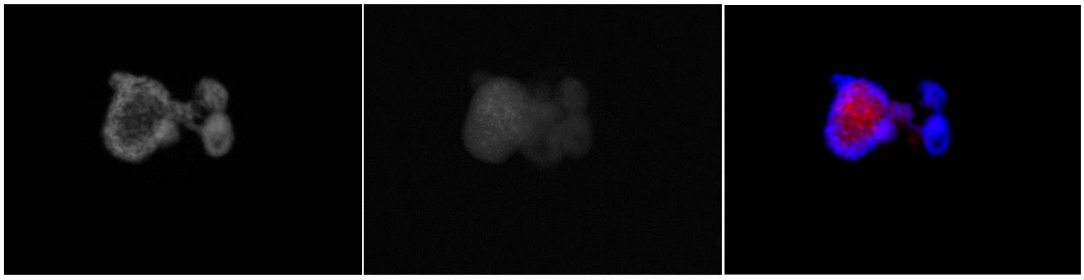
Fast-cycling Cdc42 signals to IQGAP1 to cause alveolar luminal filling and MEC invasion into the extra-alveolar environment.

As Cdc42[F28L] had previously been examined in NIH 3T3 fibroblasts grown in monolayer conditions, I was curious to see not only if this mutant would have similar effects on primary MECs growing in monolayer conditions, but also if fast-cycling Cdc42 would have any effects on the growth-arrested, apical-basal polarized MECs grown in 3D conditions. To address these questions, I used an epitope HA-tagged lentiviral system to express Cdc42[F28L] in primary

MECs grown in both monolayer and 3D culture conditions. Expression of Cdc42[F28L] in MECs could be visualized by immunofluorescence staining for HA (Figure 3.3). Monolayer cultures of MECs expressing Cdc42[F28L] displayed similar characteristics to those previously observed in NIH 3T3 fibroblasts (21), with actin-based stress fiber formation visible in the sprawling cells, many of which were multi-nucleated (Figure 3.4). These data suggested that the actions of Cdc42[F28L] are indeed similar between primary MECs and NIH 3T3 fibroblasts when the cells are cultured in monolayer conditions.

The expression of Cdc42[F28L] in MECs that had antecedently formed growth-arrested alveoli with hollowed central lumens in 3D culture commonly caused the MECs to fill this hollowed alveolar lumen, whereas expression of an empty control vector did not (Figure 3.5, compare panels a and b). Interestingly, Cdc42[F28L] also appeared to cause cell invasion into the Matrigel surrounding the alveoli (red arrowhead in Figure 3.5, panel b). Given that Cdc42 played a major role in E-Cadherin stabilization, presumably through IQGAP1, at adherens junctions in Chapter 2 (Druso, et al. 2016), and that Cdc42, IQGAP1, and Clip170 have been shown to capture and bundle microtubule (+) ends at the leading edges of migrating cells (24), I was interested in seeing whether the interaction between Cdc42[F28L] and IQGAP1 in mouse MECs played an important role in the results observed in 3D cell culture. Thus, I expressed a Cdc42[F28L,F37A] mutant, which is constitutively active but was previously shown by our laboratory to be defective in binding to IQGAP1, in the MECs grown in 3D culture. Interestingly, this Cdc42 mutant caused the luminal filling phenotype in the alveolar buds, but did not appear to cause MECs to invade into the surrounding Matrigel (Figure 3.5, panel c). As these data collectively suggest IQGAP1 as a critical binding partner of Cdc42 in MEC function, I next examined the sub-cellular localization of IQGAP1 in the MECs grown in 3D culture.

Figure 3.3. Expression of HA-tagged Cdc42[F28L] Visualized by Immunofluorescence. Immunofluorescence staining of primary MECs grown in 3D conditions displaying the expression of an HA-tagged Cdc42[F28L] vector delivered by lentivirus.



DAPI HA Merge

Figure 3.3

Figure 3.4. Constitutively-Active Cdc42 Induces Actin-Based Stress Fiber Formation and multi-nucleated cells in Primary MECs Cultured in Monolayer Conditions. Upper image shows immunofluorescence co-staining for pan-actin and DAPI in primary MECs expressing constitutively-active Cdc42[F28L]. MECs under this condition commonly displayed stress fiber formation and multi-nucleation similar to that seen in NIH 3T3 fibroblasts expressing Cdc42[F28L]. Lower image depicts schematic diagram for MECs cultured in monolayer conditions.

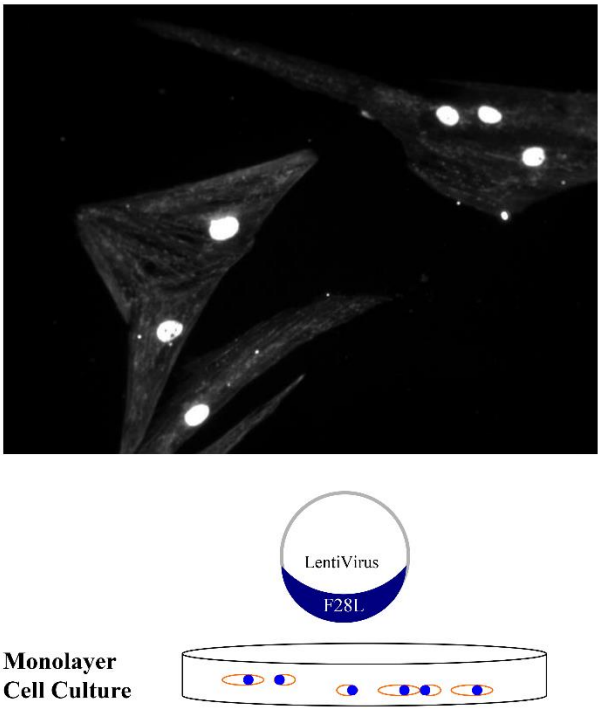
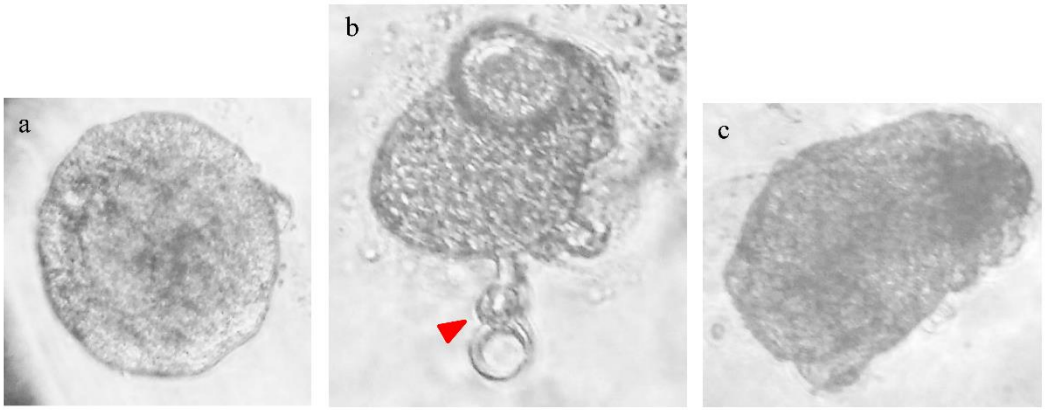


Figure 3.4

Figure 3.5. Constitutively-Active Cdc42 Causes Alveolar Luminal Filling and Invasion in an IQGAP1-dependent Manner in Primary MECs Cultured in 3D Conditions. Upper panels show phase contrast images depicting a hollowed alveolus formed by MECs expressing a control vector (panel *a*), a filled alveolus with cellular invasion into the surrounding Matrigel in MECs expressing constitutively-active Cdc42[F28L] (panel *b*, red arrowhead indicates invading cells), and a filled alveolus that does not display cellular invasion in MECs expressing constitutively-active Cdc42[F28L,F37A], which is defective in binding IQGAP1 (panel *c*). Lower image depicts schematic diagram for MECs cultured in 3D conditions.


a
b
c
Control
Cdc42 F28L
Cdc42 F28L;F37A

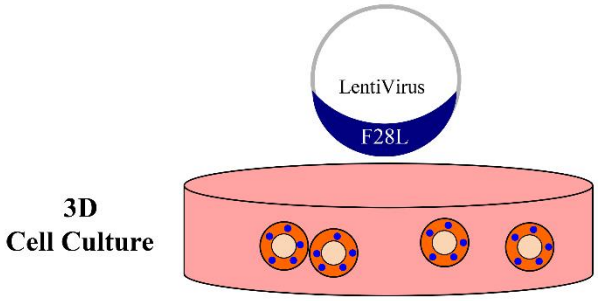

LentiVirus
F28L
3D
Cell Culture

Figure 3.5

Immunofluorescence staining of the alveolar buds grown in Matrigel showed that IQGAP1 localized to the cell-cell junctions in the mature, growth-arrested alveoli expressing a control vector (Figure 3.6, top panels). Similar to the case for the mouse mammary gland *in vivo*, these MECs also formed a laminin-rich extracellular matrix that encapsulated the alveolus and allowed the cells to attain their apical-basal polarity (Figure 3.6, bottom panels). However, within the alveoli in which Cdc42[F28L] was expressed, the localization of IQGAP1 became diffuse and was detected at early time-points (48 hours) in filopodia-like projections at the periphery of the alveolus (Figure 3.7, top left and middle panels). These IQGAP1-rich projections appeared to be invadopodia, which are formed by invasive cells and can break down the laminin-rich extracellular matrix that forms around the alveolus, allowing for cell dispersal into the surrounding stroma (2, 25). In this model system, the invadopodia represent a critical step in breast cancer progression in which abnormal, dysfunctional MECs begin to invade into their surrounding tissue and ultimately metastasize. Over time (96 hours), these invadopodia led to long extensions of cells invading into the surrounding Matrigel, with the tips of these invading cell protrusions being rich in IQGAP1 (white arrowhead in Figure 3.7, top right panel). Cdc42[F28L] was capable of causing this remarkable type of invasive behavior in the MECs grown in 3D culture, but only when it was able to signal to IQGAP1.

Fast-cycling Cdc42 disrupts E-Cadherin localization at adherens junctions and leads to its down-regulation in invading MECs.

The findings described in Chapter 2, together with the effects of Cdc42[F28L] on primary MECs grown in 3D culture, illustrate the significance of the role of Cdc42 binding to IQGAP1 in the mammary gland. Given the array of potential effects that Cdc42 signaling can have in different

Figure 3.6. IQGAP1 is Localized to Cell-Cell Contacts in Primary MECs Grown in 3D Conditions, and 3D Alveoli Are Encapsulated by a Laminin-Rich Matrix. Upper panels display immunofluorescence staining for IQGAP1 (red) in primary MECs grown in 3D conditions (nuclei in blue). IQGAP1 can be seen at cell-cell contacts in the formed alveoli. Lower panels display immunofluorescence staining for the mouse mammary gland basement membrane matrix protein laminin (red), which encapsulated each alveolus (nuclei in blue).

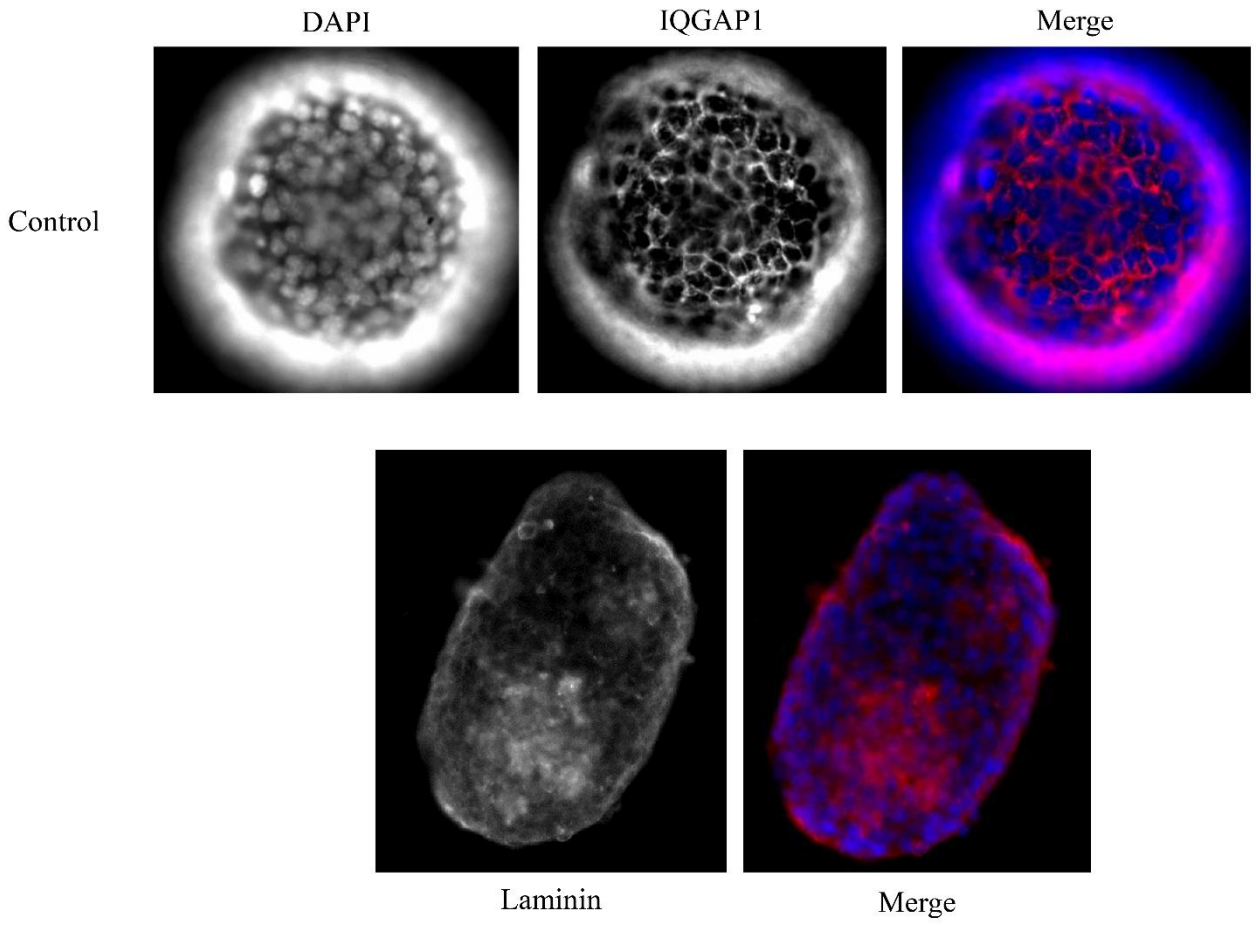


Figure 3.6

Figure 3.7. Constitutively-Active Cdc42 [F28L] Causes IQGAP1-rich Invadopodia, Cellular Invasion and Reduced E-cadherin Expression in MECs grown in 3D Culture. Upper panels display IQGAP1 (red) enrichment in invadopodia (upper left panel and white arrowhead in middle panel) that lead to cellular invasion away from the alveolus (white arrowhead in upper right panel). Lower panels display E-cadherin (red) localization at cell-cell contacts in MECs expressing control vector (white arrowheads in lower left panel), and the loss of E-cadherin expression in MECs expressing constitutively-active Cdc42[F28L] that have invaded the surrounding Matrigel (white arrowheads in lower right panel). Cell nuclei are blue.

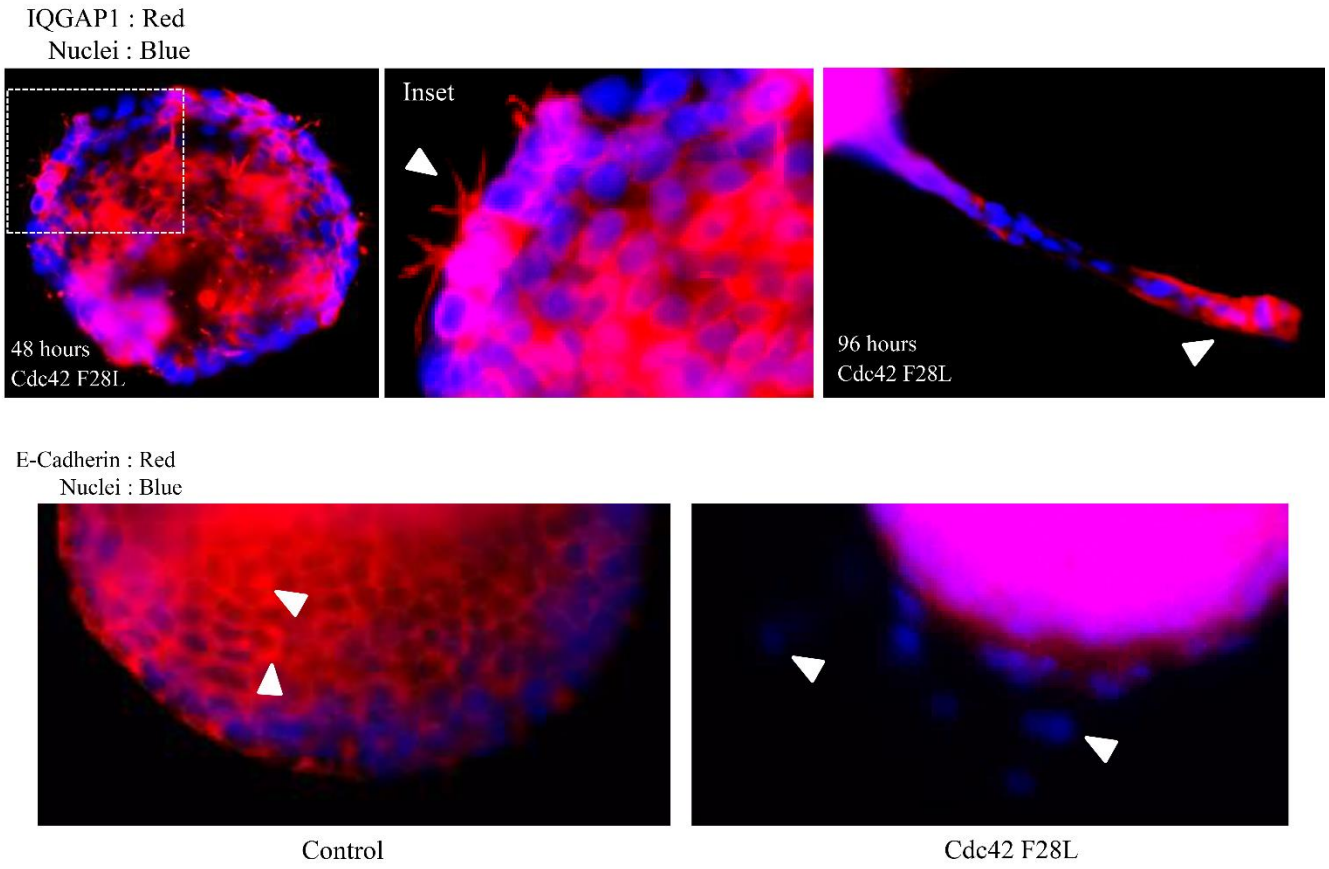

IQGAP1 : Red
Nuclei : Blue
Inset
48 hours
Cdc42 F28L
96 hours
Cdc42 F28L
E-Cadherin : Red
Nuclei : Blue
Control
Cdc42 F28L

Figure 3.7

cell types (15, 26), these data collectively suggest that the functional plasticity found in MECs from the mammary gland relies, at least in part, on Cdc42. To further investigate the link between Cdc42[F28L] signaling through IQGAP1, and the shift of MECs to an invasive phenotype, I next examined the E-cadherin-based contacts formed between the cells comprising the alveoli. Similar to the localization of IQGAP1 in the control MECs (Figure 3.6), E-cadherin was also found at the cell-cell contact points throughout the control alveoli (white arrowheads in Figure 3.7, bottom left panel). In the presence of Cdc42[F28L] expression, however, E-cadherin localization became diffuse within the MECs, again like IQGAP1, but with one striking difference. The IQGAP1-rich MECs that were seen invading out from the alveoli and into the surrounding extracellular matrix could also be visualized using a nuclear stain in this experiment, but further immunofluorescence staining showed that these invading cells did not express E-cadherin (white arrowheads in Figure 7, bottom right panel).

Taken together, these data suggest that Cdc42[F28L] is capable of altering the localization and stability of E-cadherin in primary MECs, as well as causing the cells to transition into an invasive phenotype. IQGAP1 appears to be an important effector through which this invasive phenotype is accomplished, suggesting that the previously shown mechanism of Cdc42-mediated capture of microtubules via IQGAP1 and Clip 170 (24) is present in primary mouse MECs, and may contribute to the progression of breast tumors from ductal carcinoma in-situ (DCIS) to invasive ductal carcinoma.

Discussion

In this study, I have examined the effects of aberrant Cdc42 signaling on primary MECs using two methods of cell culture. While a significant understanding of Cdc42 signaling has been obtained using monolayer cell culture systems, the advancement of 3D cell culture studies has

highlighted the important influence imparted by the extracellular environment and cell-to-cell communication on the cellular responses to mitogenic, and even oncogenic, signaling (27). With this in mind, I set out in the present study to investigate the differences in effects caused by overactive Cdc42 signaling in mammary epithelial cells grown in monolayer versus 3D culture.

I found important differences in MEC growth and survival between the two culture systems, in which the 3D condition provided morphogenesis cues to instruct the MECs to grow and organize into hollowed alveoli, whereas the monolayer condition caused the MECs to divide and migrate onto the culture dish in the absence of the cues that are provided by the tissue extracellular environment. Interestingly, together with these differences was a shortened survival of the MECs grown in monolayer conditions as compared to those grown in 3D conditions. Under monolayer growth conditions, the primary MECs divided and migrated, albeit at a slower rate compared to common immortalized cell lines, until their death at about three weeks post-plating. MECs cultured in the 3D condition did not undergo cell death after three weeks of culture, but instead grew into hollowed alveoli connected by ductal structures that remained viable during my observation period of three months post-plating. This difference suggests that the establishment of the 3D alveoli, accompanied by the formation of MEC apical/basal polarity and cell-to-cell junctions, provided a survival advantage to the cells.

Next, I investigated the potential differences in the effects triggered by a constitutively-active Cdc42 mutant, Cdc42[F28L], in MECs growing under these two cell culture conditions. Cdc42[F28L] conferred characteristics of cancer transformation onto primary MECs grown in both the monolayer and 3D culture systems, although the effects were distinct for the two culture systems. Constitutively-active Cdc42 caused MECs growing in monolayer culture to adopt traits similar to those observed in NIH 3T3 fibroblasts expressing this mutant, including actin-rich stress

fiber formation and multi-nucleated cells. However, MECs expressing constitutively-active Cdc42 in 3D culture commonly caused a filling of the alveolar luminal space and cell invasion into the surrounding extracellular matrix, traits which are characteristic of ductal carcinoma in situ and invasive ductal carcinoma of the breast, respectively (12). The invasion of MECs into the extracellular matrix began with the display of IQGAP1-rich invadopodia surrounding the laminin-rich periphery of the alveoli, which over time resulted in a large degree of cellular migration away from the primary alveoli. Normal ductal morphogenesis in this culture system involves a similar migration of cells through the 3D matrix (28), but results in the formation of a new alveolar bud composed of apical-basal polarized cells, with IQGAP1 localized to the cell-to-cell contacts (Figure 3.6). In contrast, the Cdc42[F28L]-expressing MECs also expressed IQGAP1, but were unable to form new alveoli, suggesting that the effects of constitutively-active Cdc42 in primary MECs dominated the morphogenesis cues, resulting in a morphological phenotype distinct from that normally imparted by the 3D environment.

IQGAP1 has been shown to bind to β-catenin, with this interaction disrupting the binding of β-catenin to E-cadherin (29). As the catenin family members also bind to the cytoskeleton, the interaction of β-catenin with E-cadherin ultimately results in the stabilization of E-cadherin-based cell junctions (30). This connection of the cytoskeleton to adherens junctions allows for an interactive cytoskeletal network to form between multiple epithelial cells, a characteristic that is essential for the proper cellular organization and communication that needs to occur within tissues (31). Considering this, constitutively-active Cdc42 may be serving two functions by down-regulating the epithelial characteristics of MECs growing in 3D culture. First, Cdc42[F28L] may be unable to adequately sequester IQGAP1 from β-catenin due to its rapid cycling between a signaling “on” (GTP-bound) and signaling “off” (GDP-bound) state, which over time may result

in an accumulation of IQGAP1-β-catenin complexes, and thus weakened adherens junctions. Secondly, Cdc42[F28L] binding to IQGAP1 may induce CLIP-170-mediated microtubule (+) end capture at the plasma membrane, allowing for the formation of leading edges as well as the transport of MMPs along the microtubules to those sites, thereby enabling invasive migration.

Along with the invasiveness exhibited by MECs expressing constitutively-active Cdc42, E-cadherin-based cell-cell contacts were disrupted in these MECs. As Cdc42[F28L] induced the MECs to form IQGAP1-rich invadopodia that ultimately resulted in large cellular outgrowths into the surrounding stroma, these invasive MECs also began to down-regulate their expression of E-cadherin. The ability of Cdc42 to down-regulate E-cadherin expression has been observed previously, and is a direct result of the Cdc42-dependent potentiation of EGFR-signaling activity, resulting in higher levels of activated Src (32). Active Src can phosphorylate E-cadherin molecules at the cell membrane, resulting in a higher potential for their ubiquitylation and lysosomal targeting. This process can have a significant impact over time, as the steady-state recycling and sorting of E-cadherin molecules from the plasma membrane becomes shifted towards E-cadherin lysosomal degradation. This mechanism may explain our observation of constitutively-active Cdc42 expression resulting in MECs that are invasive and fail to express high levels of E-cadherin. E-cadherin-based cell contacts are critical in the morphogenesis of many tissues and organs, and help to regulate the migration and invasion of epithelial cells (33). Breast cancer cells must down-regulate E-cadherin expression to continue their growth in the absence of appropriate growth factor signaling, so much so that E-cadherin expression has become a common therapeutic index for human breast cancer tumor grading and therapy selection (34, 35). The ability of Cdc42[F28L] to disrupt E-cadherin cell-cell junctions and stimulate the growth of primary MECs in 3D culture,

promoting their transition to an invasive phenotype, highlights the importance of the signaling roles of Cdc42 during breast cancer progression.

The capability of constitutively-active Cdc42 to confer upon primary MECs an invasive phenotype relied on this mutant's ability to bind the scaffolding protein IQGAP1. Interestingly, the expression of Cdc42[F28L,F37A], a constitutively-active Cdc42 mutant that has been shown to be unable to bind IQGAP1, still induced the primary MECs to fill the alveolar luminal space but failed to stimulate the ability of MECs to invade out of their laminin-rich extracellular matrix. These results suggest that the interaction of Cdc42 and IQGAP1 can affect the transition that takes place as breast carcinomas advance from DCIS, which display luminal filling but not invasion into the surrounding tissue, to invasive ductal carcinomas that are not only neoplastic but also invade into their stroma. Intervention of Cdc42-IQGAP1 signaling may represent a potential therapeutic strategy to prevent the progression of breast tumors to metastasize to other sites within the patient.

Here, I have taken a two-pronged approach using primary cell culture systems to address the roles of a constitutively-active Cdc42 mutant in MECs. While Cdc42[F28L] caused primary MECs to display stress fiber formation and multi-nucleation in monolayer growth, I found that constitutively-active Cdc42 also had significant effects on MECs grown in 3D culture. Considering the wealth of knowledge regarding Cdc42 signaling across multiple model systems, this context-dependent perspective of Cdc42 provides further insight into the relationship of the Cdc42 signaling found in normal-functioning mammary epithelial cells as compared to neoplastic and invasive ductal carcinomas. As epithelial cells within the mammary gland begin to lose the appropriate response to their extracellular environment, they exhibit aberrant cellular growth as they progress toward a transformed phenotype (36). Combining the previously-identified roles for Cdc42 in cell-cycle control (37), cell metabolism (38) and migration (39), with the results obtained

in this study, suggests that a complex array of signaling outputs emanate from Cdc42 in epithelial cells within human tissues. Understanding this complexity from a therapeutic perspective may require the integration of multiple model systems to observe how cells orchestrate their use of Cdc42 to achieve a variety of physiological functions, and how this changes in diseases such as breast cancer.

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Chapter 4

Conclusions and Perspectives

Due to the wide array of cellular outputs to which they can contribute, small GTPases have gained considerable attention in both developmental and disease studies during recent decades. During this time, Cdc42 has become a central signaling molecule in the regulation of a variety of cellular processes such as migration, division, differentiation and metabolism (1–6), drawing focus to the importance of the regulatory mechanisms evolved by human cells to use Cdc42 signaling for such a diverse variety of cellular activities and biological outcomes. As these types of cellular processes can lead to disease when not properly regulated, a better understanding of the mechanisms by which cells control and utilize Cdc42 signaling can contribute toward developing effective therapies.

The research presented in this thesis was centered on examining the roles of Cdc42 during normal mammary gland development, with the hope of ultimately applying that knowledge toward understanding how it might influence breast cancer progression. The initial study, presented in Chapter 2, was designed to use a loss-of-function approach to identify which of the normal functions of adult mammary epithelial cells relied on Cdc42. We found that the primary effect caused by Cdc42-deletion in the adult mouse mammary gland was a disorganization of the epithelial compartment, due to a loss of both apical-basal cell polarity and E-cadherin-based cell-cell junctions. These changes caused by the deletion of Cdc42 led to premature epithelial cell sloughing (Figure 2.7), which in turn affected the gene expression profile within the mammary gland, as evidenced by RNA sequencing analysis in Chapter 2.

Comparing this Cdc42 conditional knockout mouse phenotype with those from studies deleting Cdc42 in other tissues of the mouse brings to light a common thread in Cdc42 functioning *in vivo*, which is the maintenance of cell polarity and cell-cell communication. The loss of proper cell polarity and cell-cell communication found in different mouse tissues with targeted Cdc42-deletion led to intriguing changes in overall tissue form and function. When Cdc42 was deleted in the pancreas of mice, an organ that requires tubulogenesis comparable to that found within the mammary gland, proper lumen formation was interrupted. Interestingly, when Cdc42 was deleted in this model system, the loss of proper lumen formation was accompanied by a shift in cell fate, resulting in higher levels of acinar cell differentiation and lower levels of ductal cell differentiation (7). A change in cell fate was also observed in mice where Cdc42 was either deleted in the nervous system or the skin, resulting in altered oligodendrocyte and hair follicle differentiation, respectively (8, 9). Altogether, these findings highlight important roles for Cdc42 in orchestrating the coordination of signaling outputs in mammalian cells, such that they are able to attain proper cell fate and form functional tissues.

Conditional-deletion mouse models have provided valuable insight toward the understanding of the *in vivo* roles of Cdc42 in various tissues, and in doing so have raised further interest concerning the roles Cdc42 may have in a disease such as cancer, where proper apical-basal cell polarity and cell-cell communication are commonly lost. As Cdc42 is found to be overexpressed in certain ductal carcinomas of the breast (10), I next wanted to analyze the effects of its aberrant signaling in mammary epithelial cells to determine how the roles identified for Cdc42 in Chapter 2 may be altered. To accomplish this, I utilized the constitutively-active Cdc42[F28L] mutant, which through its hyperactive downstream signaling has been shown to confer cancer-like characteristics onto fibroblasts (11). As *in vivo* studies have suggested that, by

influencing cell polarity and cell-cell communication, Cdc42 signaling can affect the overall interactions of epithelial cells with their tissue environment, I wanted to examine the effects of hyperactive Cdc42 signaling using a model system that provides epithelial cells with an environment similar to that found in their native tissue. To this end, I cultured primary mammary epithelial cells (MECs) in not only monolayer but also 3-dimensional (3D) conditions.

When examined using a monolayer culture system, Cdc42[F28L] caused a phenotype in primary MECs resembling that observed in NIH 3T3 fibroblasts (11), with actin-based stress fiber formation and multi-nucleated cells (Figure 3.4). These data suggest that the actions of hyperactive Cdc42 signaling are indeed similar between these cell types when analyzed in a monolayer growth model system, but also bring to question how the previously-identified roles for Cdc42 *in vivo,* such as the maintenance of proper epithelial cell-cell contacts, may be affected by aberrant Cdc42 signaling.

To begin to address these types of questions, I cultured primary MECs within a 3D substratum, a condition that allowed the cells to undergo morphogenesis into hollowed luminal structures, a process that relies on cell-cell communication similarly to what is found *in vivo*. Using this model system, the expression of constitutively-active Cdc42[F28L] drove the primary MECs toward an invasive phenotype, but did so in an IQGAP1-dependent manner (Figures 3.5 and 3.7). The primary MECs expressing Cdc42[F28L] lost the normal localization of E-cadherin at cell-cell contacts (Figure 3.7), and no longer formed normal, hollowed alveolar lumens. Instead, MECs expressing Cdc42[F28L] began to abnormally fill the luminal space and invade out into the surrounding environment. These cellular characteristics are comparable to what is found in breast carcinoma development, where cancerous cells begin to fill the alveolar or ductal luminal space and, in the case of invasive ductal carcinoma, invade into the surrounding tissue and metastasize.

However, the hyperactive mutant Cdc42[F28L,F37A], which is constitutively active but unable to bind to IQGAP1, also caused a loss of proper lumen formation but did so without inducing invasive traits in the MECs. These results showed that hyperactive Cdc42 signaling was capable both of altering the normal MEC functions associated with proper alveolar morphogenesis, and of causing the cells to invade into their surrounding environment. As epithelial cell interactions with their extracellular environment can affect not only cell invasion, but also cell polarity, proliferation and differentiation, they have become highly relevant to the study of breast cancer (12, 13), further signifying the phenotype caused by Cdc42[F28L] in primary MECs.

Combining our results with those from other studies indicates that Cdc42 serves as an integral regulator in progenitor and epithelial cell sensing of the tissue environment (7, 14–16), a process that has a significant effect on the overall differentiation status of epithelial cells (17). Epithelial cells rely on extracellular cues such as E-cadherin-based contacts to maintain their form and function in tissues, whereas in cancer cells, sensing of such extracellular cues becomes altered to allow for abnormal growth and survival. An intriguing therapeutic approach might be to develop strategies that cause cancer cells to regain proper responsiveness to their native tissue environment, with the goal of inhibiting abnormal cell growth and inducing programmed cell death. To this end, studies using the ectopic expression of genes such as E-cadherin in cancer cells have shown effective inhibition of cell proliferation and tumor progression (18, 19). In Chapter 2, we show that the deletion of Cdc42 lowers the expression level of E-cadherin and shifts its localization within mammary epithelial cells, from cell-cell membrane contacts to a cytosolic distribution (Figure 2.5). The differentiation status of the Cdc42-null MECs also appeared to be affected, as they did not undergo the normal programmed cell death which is associated with mammary epithelial sloughing (Figure 2.7), but displayed the abnormal expression of Cytokeratin 8 and N-

cadherin (Figure 2.5), both of which are associated with cancerous growth within the breast (20, 21). In Chapter 3, the expression of constitutively-active Cdc42 in primary MECs also affected E-cadherin localization and expression (Figure 3.7), and this was accompanied by a shift of the cells toward an invasive phenotype. Together, these two studies link Cdc42 to the control of mammary epithelial cell function and fate. Future studies using the 3D culture of primary MECs will be required to address how altering Cdc42 signaling leads to these interesting cellular changes.

The Cdc42 effector that may be the link between the phenotypes observed in the mouse mammary gland (Chapter 2) and the primary MEC culture models (Chapter 3) is IQGAP1. This scaffolding molecule has been shown to have a significant effect on the stability of E-cadherin at epithelial cell-cell junctions (22), possibly explaining why the deletion of Cdc42 in the mouse mammary gland resulted in altered E-cadherin localization and expression (14). In the 3D culture of primary MECs, the ability of Cdc42[F28L] to cause invasiveness was also linked to IQGAP1 signaling, which might be related to the previously-identified role for Cdc42 and IQGAP1 in recruiting microtubule (+) ends to the leading edge of migrating cells (23). This type of coordination of microtubules can facilitate the transport of matrix metalloproteinases to the cell membrane, where they are capable of breaking down extracellular basement membrane proteins to allow for the type of cell invasion associated with cancer metastasis (24). Similarly to Cdc42, IQGAP1 has been shown to affect a wide array of cellular functions, including proliferation, differentiation and migration (25), and changes in its expression or function are related to cancer progression. The research presented in this thesis suggests a reliance of mammary epithelial cells on the proper regulation of Cdc42 and IQGAP1 functioning and, in the case of aberrant Cdc42 signaling, the actions of these molecules can propel MECs to lose their normal alveolar structures and invade into their surroundings. In efforts to understand the progression of breast tumors,

cancer cell metastasis has been extensively investigated, as it represents a critical stage of tumor progression that affects the prognosis and ultimate survival of breast cancer patients. As such, many studies have targeted the acquisition of invasive mobility within cancer cells (26), with the goal of inhibiting the dissemination of the primary tumor cells from the mammary parenchyma to the rest of the patient's tissues. Because of this, the functional consequences of the interaction between Cdc42 and IQGAP1 require further examination using *in vivo* and primary MEC culture models. Of particular interest would be evaluating the functional significance of the interaction between Cdc42 and IQGAP1 during *in vivo* tumorigenesis, to determine whether aberrant Cdc42 signaling can drive breast tumor formation along with metastasis in an IQGAP1-dependent manner.

In conclusion, the research presented in this thesis has contributed in part to the vast knowledge base concerning Cdc42 signaling during both normal development and cancer progression, yet questions still remain concerning how human epithelia have evolved such exquisite control over the use of this small GTPase. While the expression level, activity-state and localization of Cdc42 can together begin to describe how cells can use one gene to perform such a variety of functions, the complexity of the cell signaling found to be emanating from Cdc42 will require further research using multiple model systems. The use of several complementary model systems was essential to the progression of this research, and further integration of the data generated from these multiple model systems appears to be the next essential step in establishing a physiologically-relevant view of Cdc42 signaling in the mammary gland. As E. O. Wilson stated, "We are drowning in information, while starving for wisdom. The world henceforth will be run by synthesizers, people able to put together the right information at the right time, think critically about it, and make important choices wisely."

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