UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of E-cadherin in Mediating Epidermal Stem Cell Differentiation

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Michael Trung Pham

Committee in charge:

Professor Colin Jamora, Chair
Professor Benjamin Yu
Professor Elvira Tour

2011

The Thesis of Michael Trung Pham is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011

TABLE OF CONTENTS

LIST OF DIAGRAMS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT OF THE THESIS

The Role of E-cadherin in Mediating Epidermal Stem Cell Differentiation

by

Michael Trung Pham

Master of Science in Biology

University of California, San Diego, 2011

Professor Colin Jamora, Chair

The epidermis is a continually regenerating epithelial tissue that relies on a stringently regulated balance between proliferation and differentiation. As the proliferative stem cells within the basal layer of the epidermis transition through the suprabasal layers towards the surface of the skin, the cells lose their self-renewing ability and terminally differentiate. E-cadherin is a transmembrane

protein that mediates intercellular adhesion through binding catenin family proteins to the actin cytoskeleton and has been shown to play a role in differentiation but its importance and the precise mechanism is unknown. To determine which domain of E-cadherin is sufficient to induce differentiation, chimeric E-cadherin constructs were expressed in keratinocytes. Luciferase reporter gene assays showed that all constructs that have the p120-catenin binding induced differentiation. The p120-catenin binding domain alone was sufficient to induce differentiation suggesting that the p120-cetnin binding domain is both necessary and sufficient to induce differentiation. When p120-catenin was overexpressed, it was able to rescue the phenotype caused by the p120-catenin binding domain. Findings were validated by qPCR of endogenous keratin 10 expression. This suggests that p120-catenin sequestration by E-cadherin to the plasma membrane leading to epidermal stem cell differentiation may be due to a depletion of nuclear p120-catenin. Furthermore, of the different isoforms of p120-catenin, isoform A was determined to be the most abundant in keratinocytes.

INTRODUCTION

The Skin Structure and Homeostasis

The soft skin epidermis of vertebrates is the largest organ and vital to ensuring animal survival. Skin surrounds the outer area of the body and acts as a protective barrier that defends against dehydration, temperature change, radiation, trauma, and microorganism infection. It can also protect animals from predators through camouflage as well as increase social interaction and fitness through elaborate, distinctive decoration. In addition, the skin provides animals with the sense of touch to perceive their surroundings.

Adult skin is composed of a variety of cells originating from different embryonic origins. In mammals, the neuroectoderm cells that formed the outer surface of the embryo become the epidermis while the mesoderm derived cells just below contribute to the formation of the dermis. Mature epidermis is a stratified squamous epithelium. The squames, flat, keratinized, dead cells, make up the skin surface. The epidermis is further divided into phenotypically and transcriptionally distinct layers. The basal layer, which is the only mitotically active, organizes an extracellular matrix that comprises most of the underlying basal lamina, or basement membrane, that separates the epidermis from the dermis. A network of intermediate filaments assembled from structural proteins called keratins form heterodimers that connect to the α6β4-integrin-containing hemidesmosomes, which anchor the basal layer to the extracellular matrix as well as connect to the intercellular junctions called desmosomes. This extensive

intermediate filament network creates a mechanical framework for the epithelium (Omary et al. 2004). Integrins are cell adhesion receptors that mediate attachment between the extracellular matrix outside to the cytoskeleton inside the cell (Timpl et al. 1979).

As cells leave the basal layer and migrate towards the outer surface, the cells enter a terminal differentiation program in which the cell cycle is abandoned, mitotic activity is subsequently lost, and keratinocytes are ultimately sloughed off the skin surface. In order to preserve the stratified epidermal integrity throughout the life of an animal, there must be a pool of epidermal stem cells, basal keratinocytes that can endlessly supply terminally differentiating keratinocytes. Keratinocytes that are lost through the terminal differentiation program and tissue turnover or cell death due to injury and are replaced by stem cells (Blanpain et al. 2007).

This physiological process of maintaining a consistent number of cells specifically in the skin is called epidermal homeostasis (Blanpain and Fuchs 2009). Basal keratinocytes must induce and maintain the expression of proliferation genes (e.g. keratin 5 and keratin 14) as well as repress the expression of differentiation initiating genes (e.g. keratin 1 and keratin 10) to prevent premature terminal differentiation (Koster and Roop 2007). As the basal cells journey towards the skin surface forming the suprabasal spinous layer, granular layer, and the dead stratum corneum cells, they undergo several transcriptional changes and synthesize a new set of structural proteins. Each layer of the epidermis is distinguished by the expression of different structural

protein keratin (Fuchs and Green 1980; Sun et al. 1983; Dale et al. 1985; Fuchs 1995).

Keratins are fibrous structural proteins that assemble into bundles to form intermediate filaments. Transition from the basal layer into spinous layer results in the down regulation of intermediate filaments keratin 5 and keratin 14 expression, also used as proliferative markers, (Fuchs and Green 1980; Sun et al. 1983). Increased expression of keratin 1 and keratin 10, which are larger and create a more robust network of intermediate filaments that are interlinked with desmosomes, replace keratin 5 and 14 in spinous cells, (Fuchs and Green 1980; Sun et al. 1983); thus keratin 1 and keratin 10 have been used as markers for early differentiation in epidermis. Cell-cell junctions reinforced by the expanded cytoskeleton dampen and defend against mechanical stress.

Once cells move into the granular layer, expression of keratin 1 and 10 are down regulated and additional late-differentiation structural proteins such as filaggrin and loricrin are expressed. Filaggrin is filament-associated protein that binds to keratin fibers creating tight bundles. This process collapses and flattens cells into the cornified layer. The tightly packed, uniform layer serves as the scaffold for successive layers and reinforces the cornified envelope assembly (Dale et al. 1985). Loricrin and other proteins cross-link to strengthen this scaffold and produce the base for the lipid bilayer that generates water resistance within the skin (Steven and Steinert 1994). Although the exposed cells at the surface are sloughed away allowing for only ephemeral protection against the external environment and loss of essential bodily fluids, newly differentiated

cells continually supplant lost skin (Candi et al. 2005; Blanpain and Fuchs 2006; Koster and Roop 2007).

An increase in extracellular Ca^{2+} triggers epidermal terminal differentiation and regulates the formation of the suprabasal layers. An increasing extracellular Ca^{2+} gradient is present from the basal to granular layer in the epidermis (Menon et al. 1985; Menon et al. 1992; Elias et al. 1998). In addition, cultured primary keratinocytes exposed to increased extracellular Ca^{2+} concentration induced entry into the terminal differentiation program analogous to that of keratinocytes in vivo (Yuspa et al. 1989). The concentration of calcium required *in vitro* for increasing expression of differentiation markers keratin 1 and keratin 10 is higher than for their keratin 5 and keratin 14 predecessors; likewise, an even larger increase in calcium is needed to express late-differentiation markers loricrin and filaggrin (Yuspa et al. 1989).

Cell-cell adhesion in the epidermis: Adherens junctions and E-cadherin

Cells are connected via binding of cell adhesion molecules from one adjacent cell to the next. The stable connections that form and hold cells together within tissues play essential roles in maintaining the integrity of tissue organization and physiological function. In the epidermis, adherens junctions link cells together (Gumbiner 1996). Adherens junctions are assembled via homophilic contacts between cadherin clusters on the surface of adjacent epithelial cells. The exact mechanism that initiates cadherin clustering to form adherens junctions is still being explored. Cadherins are Ca^{2+} dependent,

homophilic, adhesion molecules that are expressed in almost all solid tissue and have been shown to be key players in cell recognition and cell sorting during development (Takeichi 1991; Takeichi 1995). Epithelial structures such as the skin express epithelial cadherin, E-cadherin. It is necessary for the skin to express E-cadherin in order for skin tissue to remain bound together and to maintain tight, polarized cell layers that can perform barrier and transport functions (Gumbiner et al. 1988).

E-cadherin is a cis homodimer characterized by long extracellular and intracellular domains. Five cadherin-type repeats make up the extracellular region of E-cadherin, called the extracellular domains, which are bound together by Ca^{2+} ions (Gumbiner 2005). The intracellular region consists of binding sites for cytoplasmic plaque proteins, the catenins. (Gumbiner 1993). To be functionally active, E-cadherin needs to form core protein complexes consisting of a parallel E-cadherin dimer and interacting with catenin family proteins as well as the actin cytoskeleton (Gumbiner et al. 1988; Kemler et al. 1989; Gumbiner 2005).

Calcium stabilizes and stiffens the extracellular domains of E-cadherin changing the conformation from globular to more rod-like. The rod-like structure expresses adhesive function and participates in homophilic binding (Ringwald et al. 1987; Pokutta et al. 1994). An increasing extracellular Ca^{2+} gradient is present from the basal to granular layer in the epidermis (Menon et al. 1985; Menon et al. 1992; Elias et al. 1998). This gradient correlates with the increasing expression of stabilized E-cadherin from the basal layer towards the skin surface.

Catenin family proteins: Regulators of E-cadherin and the Adherens Junctions

Although much of how it occurs is still unknown, there is an array of evidence that shows catenin family protein interaction with E-cadherin regulates E-cadherin clustering and facilitates cell-cell adhesion, cytoskeletal anchoring, and signaling (Takeichi 1995; Yap et al. 1997; Yap et al. 1998; Gumbiner 2000; Yagi and Takeichi 2000). β-catenin, well known for its role as a signal transduction molecule that mediates signaling in the Wnt signalling pathway (Moon et al. 2002), is an armadillo repeat domain protein and binds directly to the cytoplasmic tail of E-cadherin (Fig. 2) (Reynolds et al. 1992). The cytoplasmic tail of E-cadherin is stabilized after β-catenin binds the distal part of the intracellular domain (Huber et al. 2001). β-catenin associates with E-cadherin in the endoplasmic reticulum and is necessary for proper targeting and translocation to the plasma membrane (Chen et al. 1999; Lock and Stow 2005). At the plasma membrane, β-catenin recruits α-catenin to the distal part of the cadherin cytoplasmic domain.

The binding of β-catenin is essential to full adhesive function since it initiates linkage of α-catenin to E-cadherin (Fig. 2), which is necessary for adherens junction formation hence cadherin-mediated cell adhesion (Benjamin and Nelson 2008). It has been shown that deletion of the β-catenin binding domain inactivates E-cadherin adhesion indicating that β-catenin is necessary for proper cell-cell contact via E-cadherin (Ozawa and Kemler 1998). *In vitro* studies

have shown that α -catenin has actin-binding activity, which suggests that it can directly bind actin filaments and physically link E-cadherin to the actin cytoskeleton (Rimm et al. 1995). However, it is also possible that α-Catenin mediates linkage to the actin cytoskeleton through the binding of other actin binding proteins such as vinculin, -actinin, formin, zonula occludens protein 1, and afadin (Knudsen et al. 1995; Watabe-Uchida et al. 1998; Kobielak and Fuchs 2004). Data suggesting which or any of the mechanisms for E-cadherin linkage to the actin cytoskeleton have not been concluded.

The proximal region of the cytoplasmic domain associates with p120-catenin (Reynolds et al. 1994; Shibamoto et al. 1995; Yap et al. 1998). p120-catenin is also an armadillo repeat domain protein that binds directly to the cytoplasmic tail of E-cadherin (Fig. 2) (Reynolds et al. 1992). There is no evidence for p120-catenin mediating a link to the actin cytoskeleton. However, when p120-catenin interacts with E-cadherin, cell-cell adhesion is stabilized and the degradation and endocytosis of E-cadherin is prevented (Davis et al. 2003; Xiao et al. 2005). This suggests that p120-catenin is a positive master regulator of E-cadherin that protects E-cadherin from endocytotic machinery. Remarkably, p120-catenin is able to influence the clustering of E-cadherin independent of β-catenin (Aono et al. 1999). p120-catenin binds directly to RhoA, a GTPase involved in structural remodeling, (Anastasiadis et al. 2000) suggesting that recruitment of RhoA to E-cadherin may play a role in E-cadherin clustering, but this has yet to be explored.

Multiple isoforms of p120-catenin are expressed in most cells types, some of which are tissue specific, and are derived from the alternative splicing of a single gene (Mo and Reynolds 1996; Keirsebilck et al. 1998). N-terminal splicing events use four different translation start site ATGs leading to the expression of four p120-catenin isoforms, 1-4. It has been determined that epithelial cells normally express p120-catenin isoforms 3 and 4. Also, isoforms are derived from combinations with alternatively used exons A (exon 18) and B (exon 20); thus, isoforms may contain A and B, exclusively one or the other, or neither (Mo and Reynolds 1996; Keirsebilck et al. 1998; Aho et al. 2002). The actual function of exon A has not been uncovered, but exon B is a nuclear export signal suggesting p120-catenin can shuttle in and out of the nucleus to perform its functions (van Hengel et al. 1999).

E-cadherin influence on epidermal homeostasis

Whereas the morphogenetic changes that are associated with stratification have been well studied, the molecular mechanisms that orchestrate skin differentiation remain poorly understood (Koster and Roop 2007). Mouse genetics have identified multiple signaling pathways that are essential for proper epidermal stratification (Blanpain and Fuchs 2006; Koster and Roop 2007). E-cadherin is thought to mediate intercellular adhesion in the mammalian epidermis and in hair follicles as the adhesive component of adherens junctions at the plasma membrane.

Upon deletion of E-cadherin in mouse epidermis, epidermal differentiation is altered (Young et al. 2003; Tinkle et al. 2004). Loss of E-cadherin in the epidermis led to a thickening of the entire epidermis marked by a robust increase in keratin 6 expression indicative of hyperproliferation (Young et al. 2003; Tinkle et al. 2004). In addition, epidermal stem cell marker keratin 14 was more easily distinguished due to the larger basal cells whereas late differentiation marker loricrin was downregulated and fillagrin less processed (Young et al. 2003; Tinkle et al. 2004). Furthermore, nuclear staining of proliferative marker Ki67 revealed that there was an increase in proliferating cells of the basal layer in the knockout mouse as compared to the wildtype (Tinkle et al. 2004). These data suggest that E-cadherin is necessary for cell differentiation without which the homeostatic balance shifts to favor proliferation.

A transgenic mouse overexpressing E-cadherin in the epidermis was generated resulting in the completely opposite phenotype. Staining of transgenic mice back skin revealed expression of proliferative markers keratin 5 in a thinner basal layer as compared to wild-type mouse (Fig. 3B). Surprisingly, expression of early epidermal differentiation marker keratin 1 and late epidermal differentiation marker loricrin co-localized with keratin 5 in the basal layer of transgenic mouse back skin (Fig. 3B). The significant reduction in proliferating cells (Fig. 3C) and atypical expression of differentiation markers in the basal layer of the epidermis suggested that the overexpression of E-cadherin prompted premature entry of basal keratinocytes into the terminal differentiation pathway. Moreover, when E-

cadherin was overexpressed *in vitro,* cancer cell line SW480 loss its proliferative capacity (Gottardi et al. 2001).

These findings create a robust list of arguments for adhesion, specifically E-cadherin, to possibly influence stem cell fate leading to differentiation. Here, I examine E-cadherin and its role in regulating stem cell fate. Basal keratinocytes are epidermal stem cells that necessarily need to decide whether to self-renew to maintain the stem cell pool or differentiate in order to replace and repair lost tissue. To understand which domain(s) of E-cadherin are sufficient to initiate epidermal differentiation, chimeric constructs of E-cadherin were transiently transfected into basal keratinocytes. The extracellular domains of E-cadherin were dispensable. Interestingly, p120-catenin binding domain alone was sufficient to induce differentiation. Further experiments revealed that p120-cateninA isoform was the most abundant in keratinocytes and E-cadherin mediated differentiation may be caused by sequestration of p120-catenin to the plasma membrane.

RESULTS

Transfection of primary murine keratinocytes with E-cadherin induces differentiation

Overexpression of E-cadherin *in vivo* within the basal layer of murine epidermis suggested E-cadherin induced primary keratinocytes to prematurely enter into the terminal differentiation pathway. In order to determine if these findings were reproducible *in vitro*, E-cadherin was cotransfected with differentiation marker keratin 10 luciferase reporter into primary murine keratinoctyes. An increase in luciferase reporter indicated the cells differentiated. Cultured primary keratinocytes were seeded in triplicate into a 24-well plate and allowed to settle over a four hour period prior to transfection. Empty, pCDNA3 vector was cotransfected with keratin 10 luciferase reporter into two triplicate sets as control. The following day, 1.2 mM calcium final volume was added to 1 set of vector transfected cells as positive control to differentiate cells. After 24 hour calcium treatment, luciferase measurements were taken for each condition using *Promega* Dual-*Luciferase*® Reporter *Assay kit*. E-cadherin transfected cells showed increased luciferase expression comparable to those of calcium-switched keratinocytes (Fig.4B). Extensive luciferase assays of calcium-switch positive control established a minimum 1.5 fold in keratin 10 luciferase reporter activity denoted induction into early differentiation.

To determine which domain or domains of E-cadherin are sufficient to induce terminal differentiation of murine primary keratinocytes, chimeric E-

cadherin constructs (Fig. 4A) were cotransfected with the same luciferase reporter under the same conditions as previously mentioned. There are seven constructs in total: full length E-cadherin; E-cadΔcyto which lacks the intracellular domains that bind the catenin family members but retains extracellular adhesion; E-cadΔp120BD which is full length E-cadherin with a mutated p120-catenin binding site; E-cad/α-cat which is truncated full length E-cadherin lacking the β-catenin binding domain but fused to α-catenin in order to maintain adhesive properties via linkage to the actin cytoskeleton; IL2R/cyto which is interleukin 2 receptor α fused to the intracellular domains allowing interaction with the catenin family proteins but lacking extracellular cell-cell adhesion; IL2R/p120 BD which is the p120-catenin binding domain alone fused to interleukin 2 receptor α; and IL2R/β-cat BD which is the β-catenin binding domain alone fused to interleukin 2 receptor α (Fig. 4A). All constructs that have the extracellular domain are HA tagged to distinguish them from endogenous E-cadherin. As for all the intracellular domain constructs lacking the extracellular domain, those are fused to IL2Rα in order to drive transport of the construct to the plasma membrane.

Results indicated that the extracellular domains responsible for adhesion, depicted by the Ecad/Δcyto construct, are dispensable for inducing differentiation (Fig.4B). Alternatively, the cytoplasmic tail of E-cadherin, IL2R/cyto, was able to induce differentiation indicating the regulation of differentiation is linked to the intracellular domains and catenin protein signaling (Fig.4B). Ecad/α-cat and IL2R/p120-cat BD, were also able to induce differentiation (Fig.4B). On the other

hand, full length E-cadherin with mutated p120 binding site and the β-catenin binding domain constructs were unable to induce differentiation (Fig.4B).

Table 1. Primers used for isoform detection.

PRIMER	SEQUENCE
Exon 17 Forward	ACTCTCCCCCTCATTGACCGG
Exon 18 Forward	TGACCGGGAAGAAATTCCAATGAGC
Exon 20 Reverse	TCCTCCAAAGATTCCTGCCCCTC
Exon 21 Reverse	TATGGAGCAGGTAGAGTGGCGC

In order to investigate whether exogenous p120-catenin can rescue the phenotype, primers were designed (Table 1) to determine what alternatively spliced isoforms are present. PCR analysis revealed that all 4 alternatively spliced isoforms were present in mouse keratinocytes, with p120A lacking the nuclear export signal being the most abundant, followed by p120 lacking both exon A and B(Fig. 7). Both full length p120-catenin and p120-cateninA isoform lacking the nuclear export signal were cloned from cDNA into pCDNA3 vector and tagged with T7.

Cotransfection of p120-catenin binding domain construct with full length p120-catenin construct, p120(T7), to increase expression of p120-catenin protein, rescued the effect caused by the p120-catenin binding domain illustrated by the keratin 10 luciferase reporter (Fig.4B). qPCR analysis of IL2R/p120 BD and p120(T7) also recapitulated these results (Fig. 6) and validated the findings.

Interestingly, cotransfection of p120-catenin binding domain construct with p120-catenin lacking the nuclear export signal, p120ΔB(T7), was also able to rescue the phenotype. This finding suggested that localization of p120-catenin may be responsible for E-cadherin mediated differentiation.

To examine whether localization of exogenous p120-catenin affects its ability to rescue the differentiation phenotype, keratinocytes were transfected with p120(T7) and treated with high calcium to induce differentiation. p120(T7) in both low and high calcium was not present in the nucleus at all (Fig. 8A) due to the nuclear export signal. p120-catenin lacking the nuclear export signal has been shown to be present in both the nucleus and the cytosol as well as colocalize with E-cadherin (van Hengel et al. 1999).

p120(T7) was also cotransfected with IL2R/p120 BD. Although supposed to be localized to the plasma membrane, IL2R staining shows that p120-catenin binding domain is present throughout the cell (Fig. 8B). It does appear, nevertheless, that p120(T7) co-localized with IL2R/p120 BD (Fig. 8B). These data suggest that perhaps E-cadherin sequestration of p120-catenin from the to the plasma membrane may disrupt p120-catenin binding to other partners, such as RhoA (Anastasiadis et al. 2000) or Kaiso (Zhang et al. 2010) that require binding to the armadillo repeat, or deplete nuclear p120-catenin leading to downstream transcriptional changes.

FIGURES

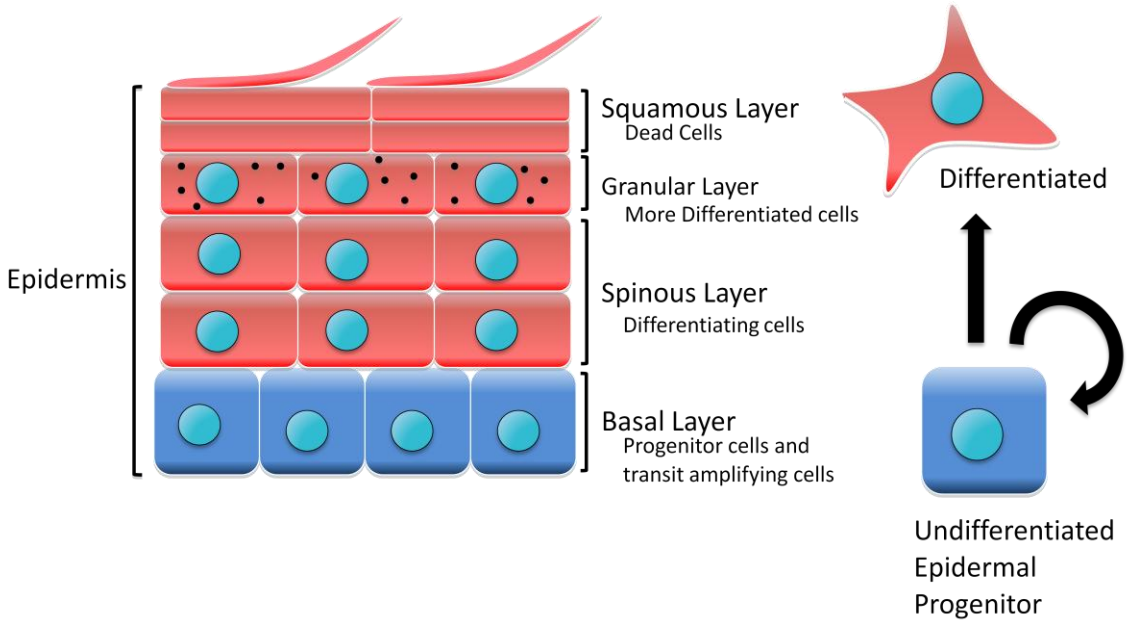


Figure 1. Anatomy of the epidermis. The epidermis is separated into four layers distinguished by morphology and expression of distinct biochemical markers. As cells progress from the basal layer toward the skin surface they begin a process of terminal differentiation.

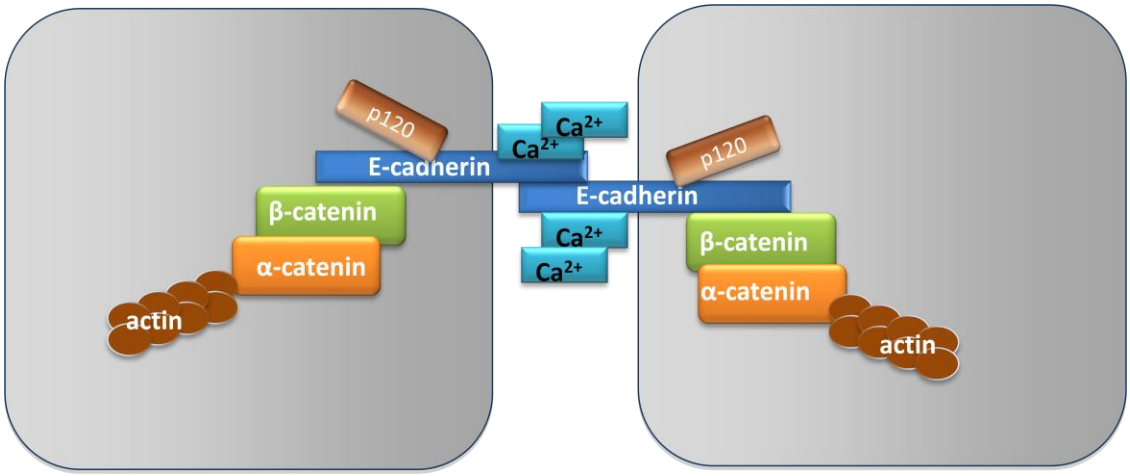
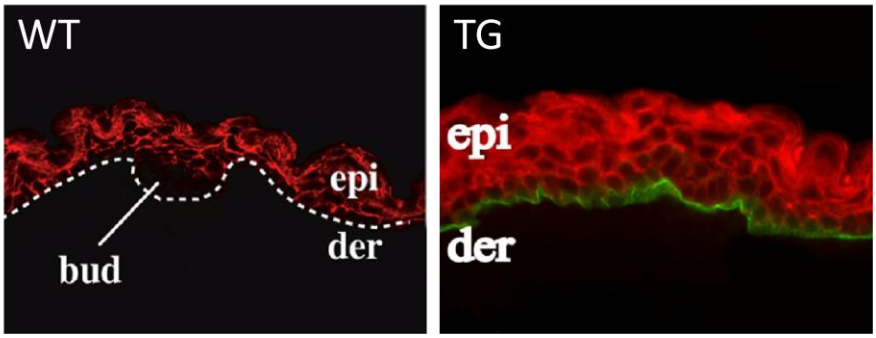


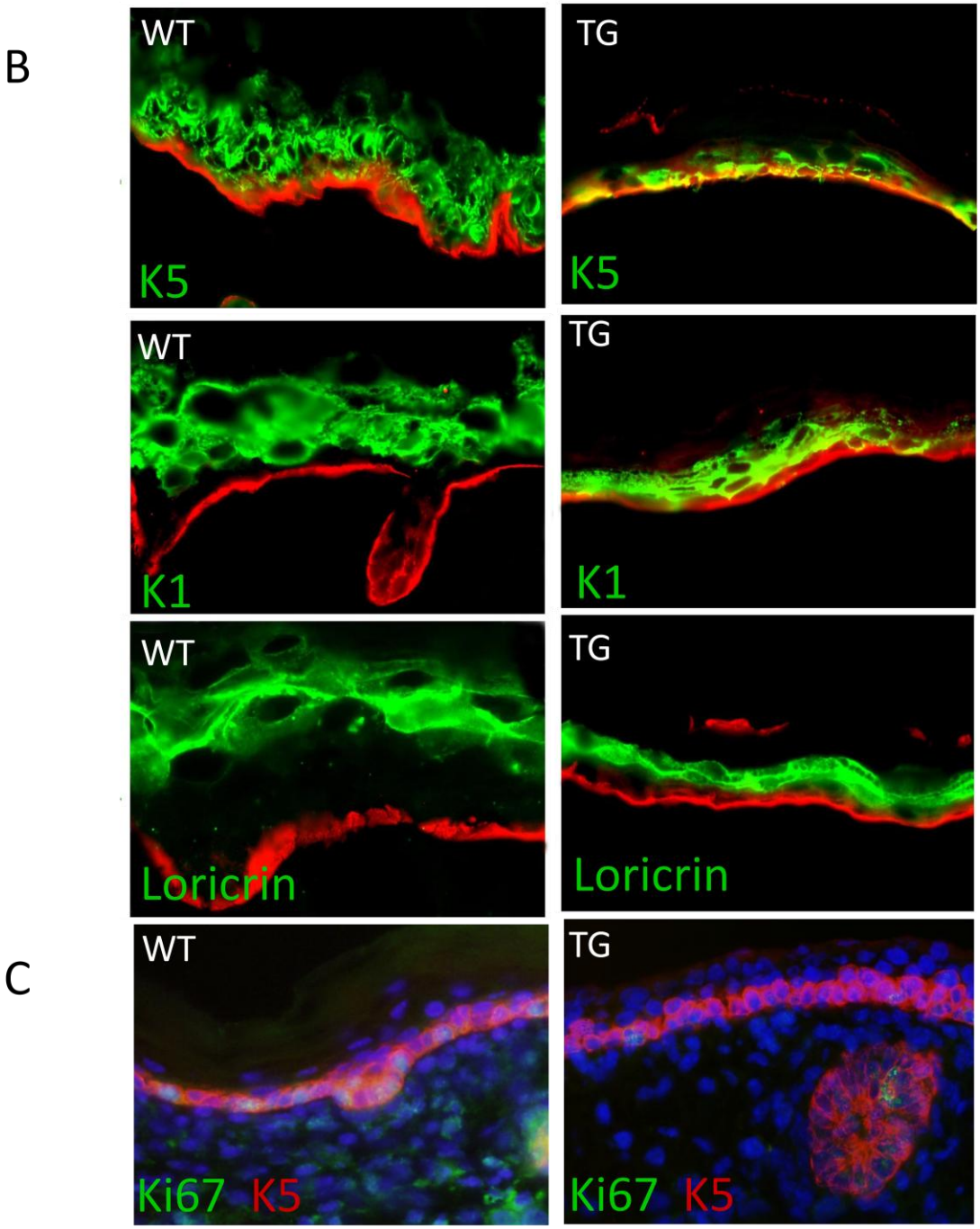
Figure 2. Basic structural components of the adherens junctions. E-cadherin is at the core of an adherens junction and is separated into extracellular and intracellular domains. The extracellular domains are stabilized by Ca^{2+} and facilitate homophilic adhesion. The intracellular domains complex with catenin family members. The intracellular region is stabilized by p120-catenin binding. β-catenin binding localizes E-cadherin at the plasma membrane. β-catenin recruits α-catenin, which links to the actin cytoskeleton.

A



Jamora unpublished.

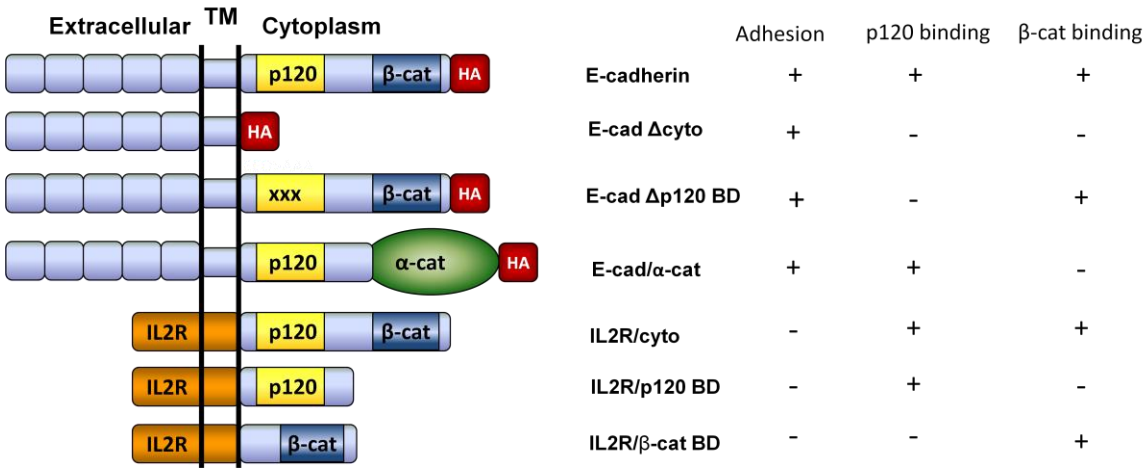
Figure 3. K14-E-cadherin(HA) transgenic mouse back skin A) E-cadherin overexpression disrupts the gradient of adhesion observed in wild type epidermis by forcing uniform levels of adhesion throughout the epidermis. E-cadherin is labeled in red and basement membrane is labeled in green or dotted line, which separates the epidermis (epi) from the dermis (der). B) E-cadherin induces early entry of epidermal stem cells into the differentiation pathway. Basement membrane is labeled in red. Proliferative or differentiation marker indicated labeled in green. C) E-cadherin overexpression reduces proliferation in basal cells. Proliferative markers labeled as indicated. Dapi blue staining of nucleus.



Jamora unpublished.

Figure 3 continued.

A



Adapted from Gottardi 2001 JCB

Figure 4. Keratin 10-luciferase reporter assays. A) E-cadherin chimeric constructs. Constructs with extracellular domains tagged with HA to distinguish from endogenous. Intracellular domain constructs fused with interleukin 2 receptor alpha. (+) presence, (-) missing. B) Cultured epidermal stem cells were co-transfected with E-cadherin constructs and a luciferase reporter. Representational data.

B

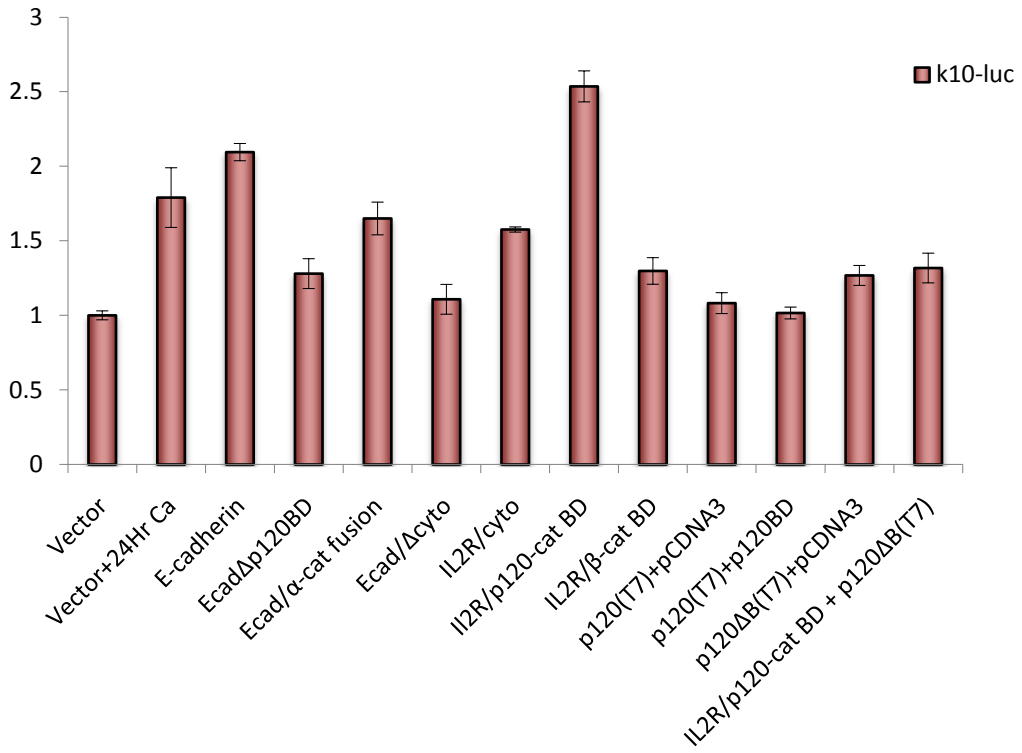
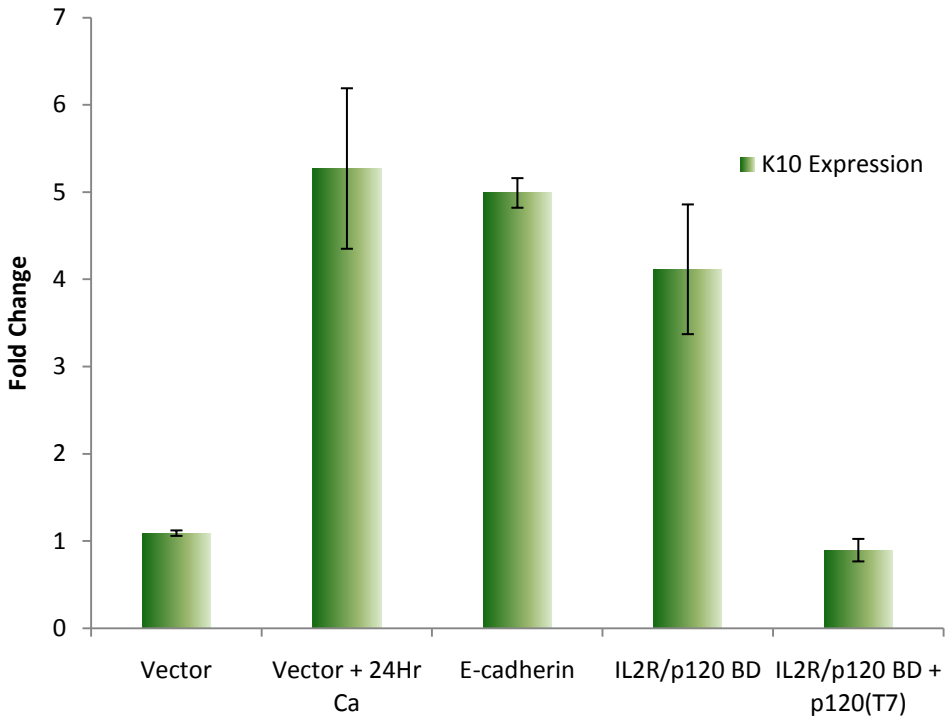


Figure 4 continued.



Lasse unpublished.

Figure 5. qPCR Analysis of endogenous keratin 10 expression. Murine basal keratinocytes were transiently transfected and FAC sorted prior to qPCR. p120-binding domain elevates keratin 10 expression indicative of entry into terminal differentiation pathway. Error bars are standard error of the mean.

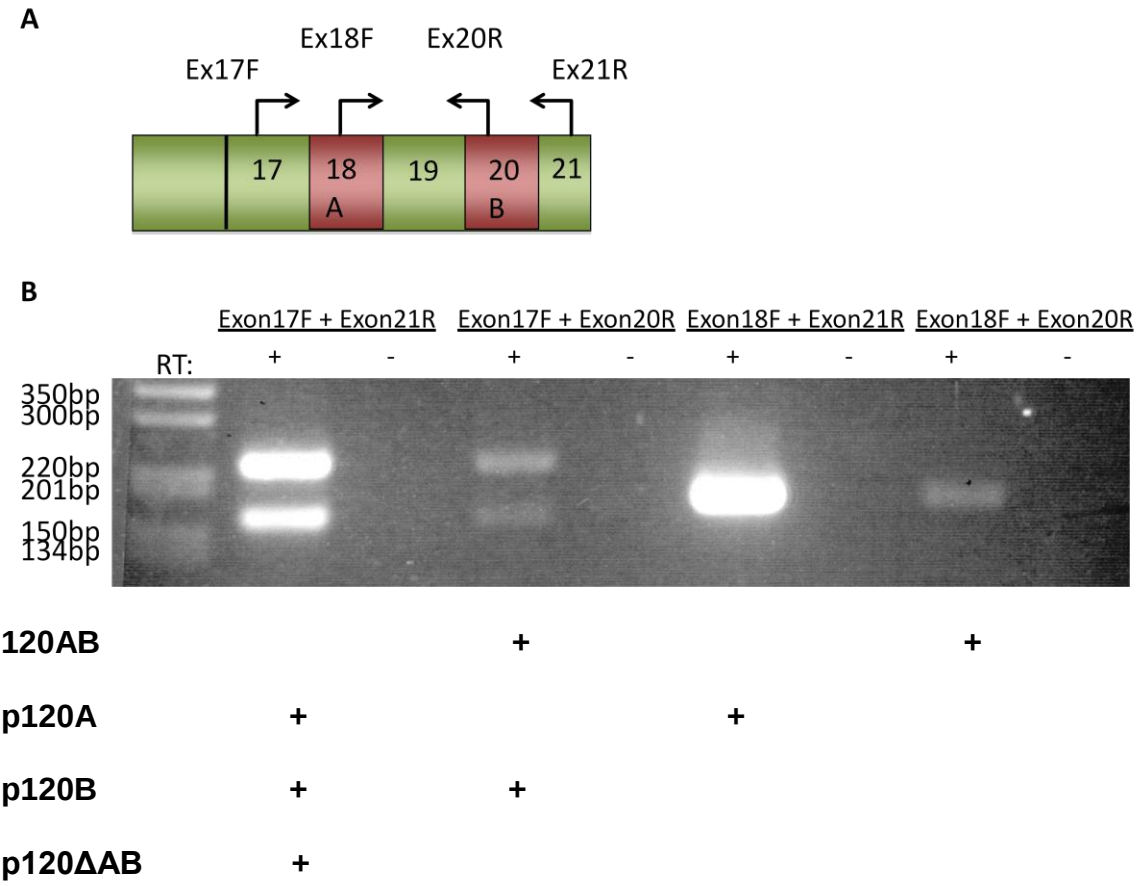


Figure 6. p120-catenin isoforms expression. A) Schematic diagram of splice variants and primer placements. B) PCR analysis of p120-catenin from mouse cDNA library reveals that p120AB (full length), p120A (p120ΔB), p120B (p120ΔA), and p120ΔAB isoforms are all present in mouse keratinocytes. Expected sizes for Exon 17 to Exon 21: AB, 321bp; A, 234bp; B, 258 bp; ΔAB, 171. (+) presence, (-) or ( ) absence

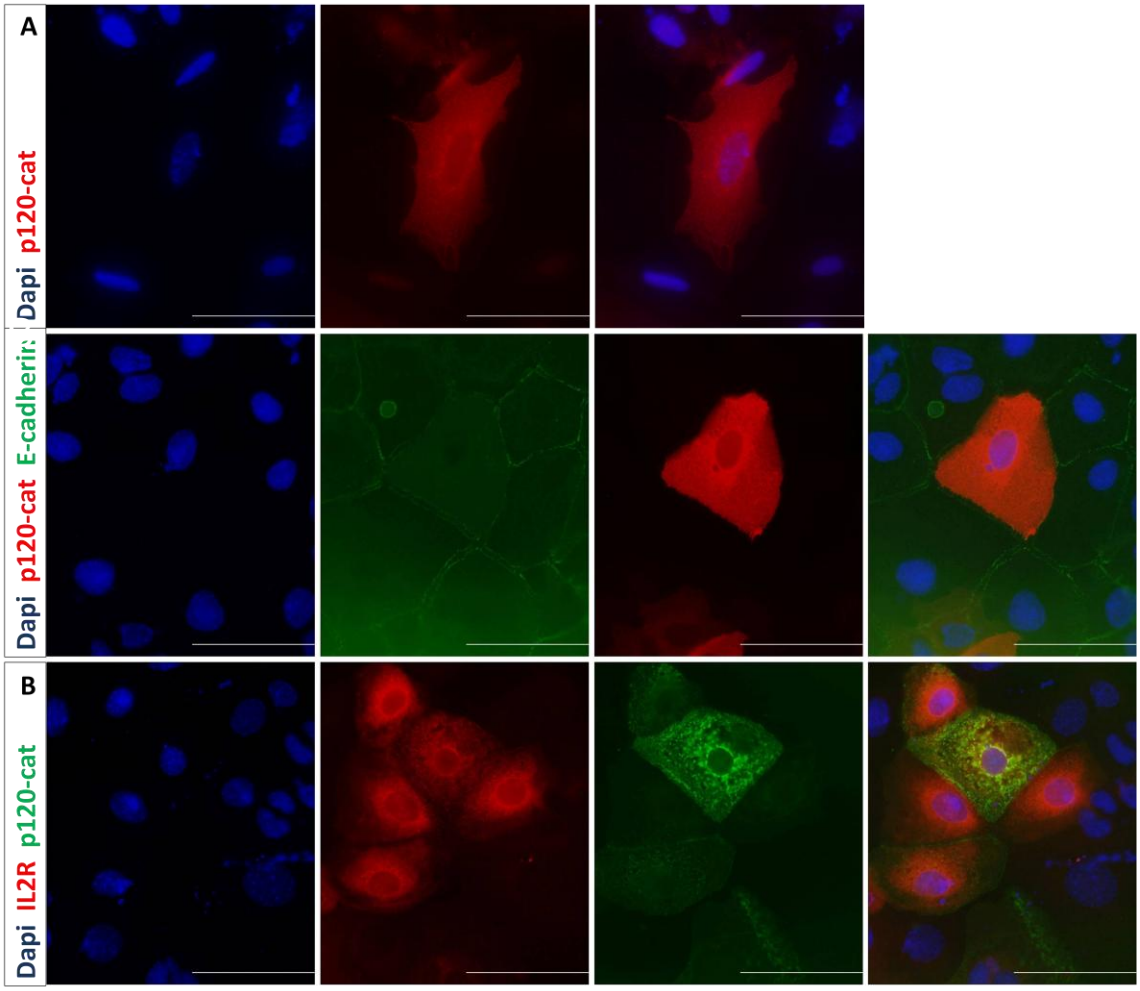


Figure 7. Localization of p120-catenin. A) Top row shows p120-catenin transfected mouse keratinocytes at low calcium, bottom row at high calcium. At both low and high calcium, p120-catenin(T7) is present throughout the cytoplasm but not within the nucleus. B) p120-catenin binding domain and p120-catenin(T7) co-localize within the cytoplasm and at the plasma membrane; no nuclear staining.

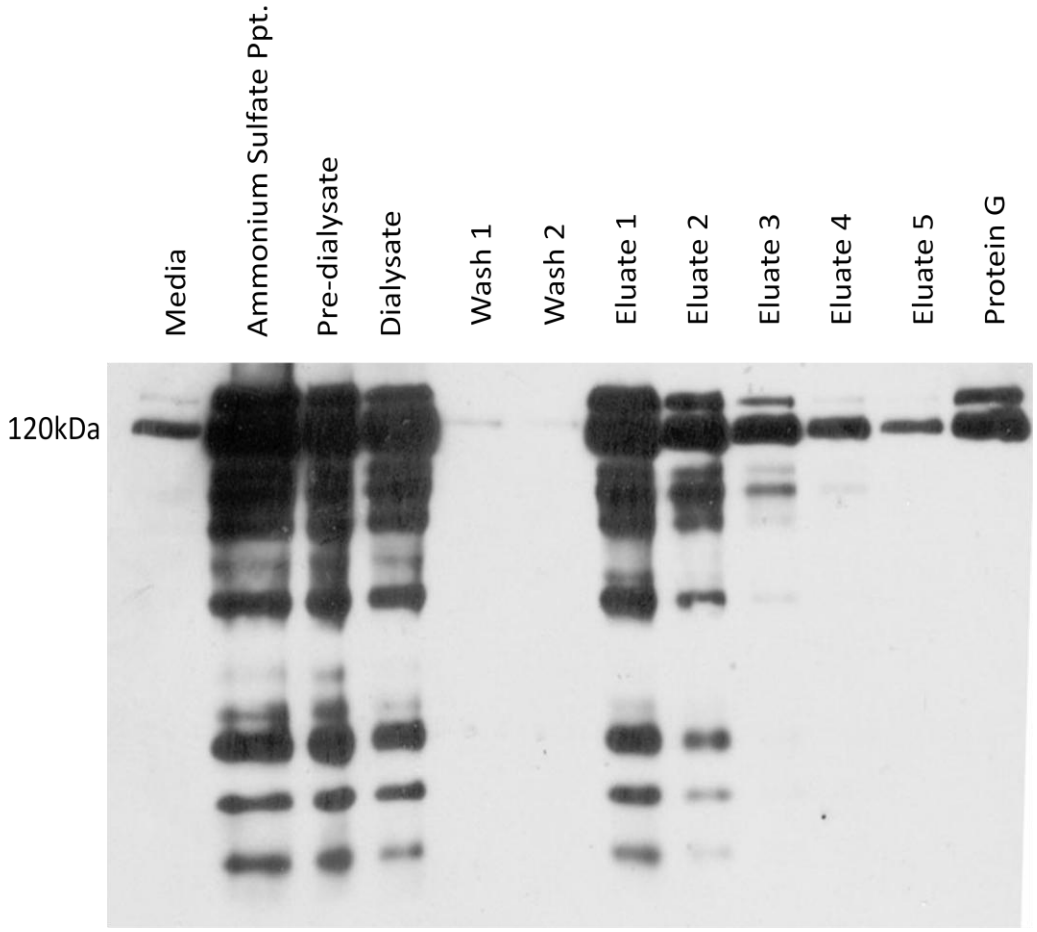


Figure 8. Immunodetection of Ecad:Fc protein from purification. Detection of Ecad:Fc with anti-human IgG. Expected band size: ~120kDa.

DISCUSSION

Cadherins are Ca^{2+}-dependent cell–cell adhesion receptors whose function requires association with the actin cytoskeleton through cytoplasmic proteins (Takeichi 1995; Yap 1998). E-cadherin has been shown to be important in development and morphogenesis.(Gumbiner 2005; Marthiens et al. 2010). Here, transient transfection of primary murine keratinocytes has been able to demonstrate a novel role for cell adhesion in determining stem cell fate. Specifically, it is shown that the p120-catenin binding domain of E-cadherin is sufficient to induce keratinocyte entry into the terminal differentiation pathway. p120-catenin is a master regulator of E-cadherin and functions to prevent E-cadherin recycle and degradation (Davis et al. 2003). Cotransfection of p120-catenin binding domain with p120-catenin in keratinocytes was able to preserve basal levels of differentiation gene expression suggesting that sequestration of p120-catenin to the plasma membrane by E-cadherin functions not only to stabilize E-cadherin but leads to the induction of terminal differentiation.

p120-catenin sequestration by E-cadherin induces differentiation

Previously, E-cadherin ablation in murine epidermis was shown to induce hyperproliferation, downregulate the expression of differentiation genes, and expand proliferative gene expression into outer layers (Young et al. 2003; Tinkle et al. 2004). Upon overexpression of E-cadherin, the opposite was observed with a thinner basal layer and coexpression of differentiation with proliferative markers

in the basal layer suggesting premature entry into the terminal differentiation pathway (Jamora unpublished). Together, this suggests a role for E-cadherin in regulating the balance in stem cell self-renewal and differentiation. Overexpression of p120-catenin binding domain of E-cadheirn in keratinocytes was sufficient to induce differentiation. However, cotransfection of IL2R/p120 BD with full length p120-catenin or p120-catenin lacking the nuclear export signal was able to rescue the differentiation phenotype.

In human epithelial tissue, p120 isoform 3 and 4 have been shown to be expressed preferentially (Mo and Reynolds 1996; Keirsebilck et al. 1998). Here, it is found that the most prevalent alternatively spliced variant is p120A (Fig. 7). Surprisingly, although lacking the nuclear export signal, isoform A has been shown to localize in both the nucleus and the cytosol (van Hengel et al. 1999). The data suggests that exogenous p120-catenin may prevent the depletion of endogenous p120-catenin and force nuclear localization of endogenous p120-catenin resulting in the inhibition of E-cadherin induced differentiation.

While the exact mechanism is poorly understood, RhoA, a GTPase part of the Ras superfamily involved with regulation of cell division, is required for E-cadherin clustering and directly binds to p120-catenin in the cytosol (Braga et al. 1997; Takaishi et al. 1997; Anastasiadis et al. 2000). p120-catenin may bridge or facilitate E-cadherin and RhoA interaction leading to E-cadherin clustering and other downstream events affecting cell division. Furthermore, p120-catenin has also been determined to act as a transcriptional repressor by directly binding Kaiso (Zhang et al. 2010). Though the significance of this documented interaction

is unclear, reduced levels of p120-catenin expression has been linked to aggressive tumorigenesis (Reynolds and Roczniak-Ferguson 2004; Davis and Reynolds 2006; van Hengel and van Roy 2007). It was also shown that the binding of p120-catenin to E-cadherin, RhoA or Kaiso are mutually exclusive events because they require binding to p120-catenin's armadillo repeat domains. Given that a large pool of p120-catenin is localized in the cytosol, it is possible that sequestration of p120-catenin to E-cadherin at the plasma membrane may deplete nuclear p120-catenin or disrupt p120-catenin from binding to other partners causing changes in downstream events leading to differentiation (Diag. 1).

E-cadherin as a metastasis repressor

Epithelial-mesenchymal transition is characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility believed to be involved in developmental processes. Loss of E-cadherin has been correlated with tumor invasion in the majority of epithelial tumors and is thought to be related to this developmental program (Cavallaro et al. 2002; Cavallaro and Christofori 2004a). E-cadherin's role as a metastasis repressor has been well established (Di Croce and Pelicci 2003; Cavallaro and Christofori 2004a). For instance, loss of E-cadherin in epithelial cells abolishes cell-cell contact and leads to increased motility (Di Croce and Pelicci 2003; Margulis et al. 2005). On the other hand, overexpression of E-cadherin in cancer cell lines prevents invasiveness

Recent studies have shown that conditional targeting of p120-catenin in epithelial tissue leads to tumor growth and increases tumor invasiveness (Yanagisawa et al. 2008; Smalley-Freed et al. 2011). Due to its E-cadherin stabilizing function, loss of p120-catenin would naturally lead to the recycling and degradation of E-cadherin. In this study, p120-catenin was found to play a role in regulating differentiation, thus leading loss of E-cadherin would lead to an imbalance of self-renewal. (Frixen et al. 1991; Vleminckx et al. 1991; Cavallaro and Christofori 2004b). It has further been shown that the β-catenin binding domain of E-cadherin responsible for sequestration of β-catenin from the nucleus to the plasma membrane inhibits proliferation (Gottardi et al. 2001). Collectively, these data suggest that loss of E-cadherin expression can contribute to down regulation of the p120-catenin pathway and upregulation of the β-catenin pathway in epithelial cancers.

β-catenin has been widely studied for its role in cell growth and relation in cancer. However, p120-catenin's role in regulating cell fate has been much unappreciated. The discovery of p120-catenin's novel role as a key element to epithelial cell differentiation and p120-catenin's implications with cell growth establishes it as an interesting oncogene worthy of dissection in other tissues as well.

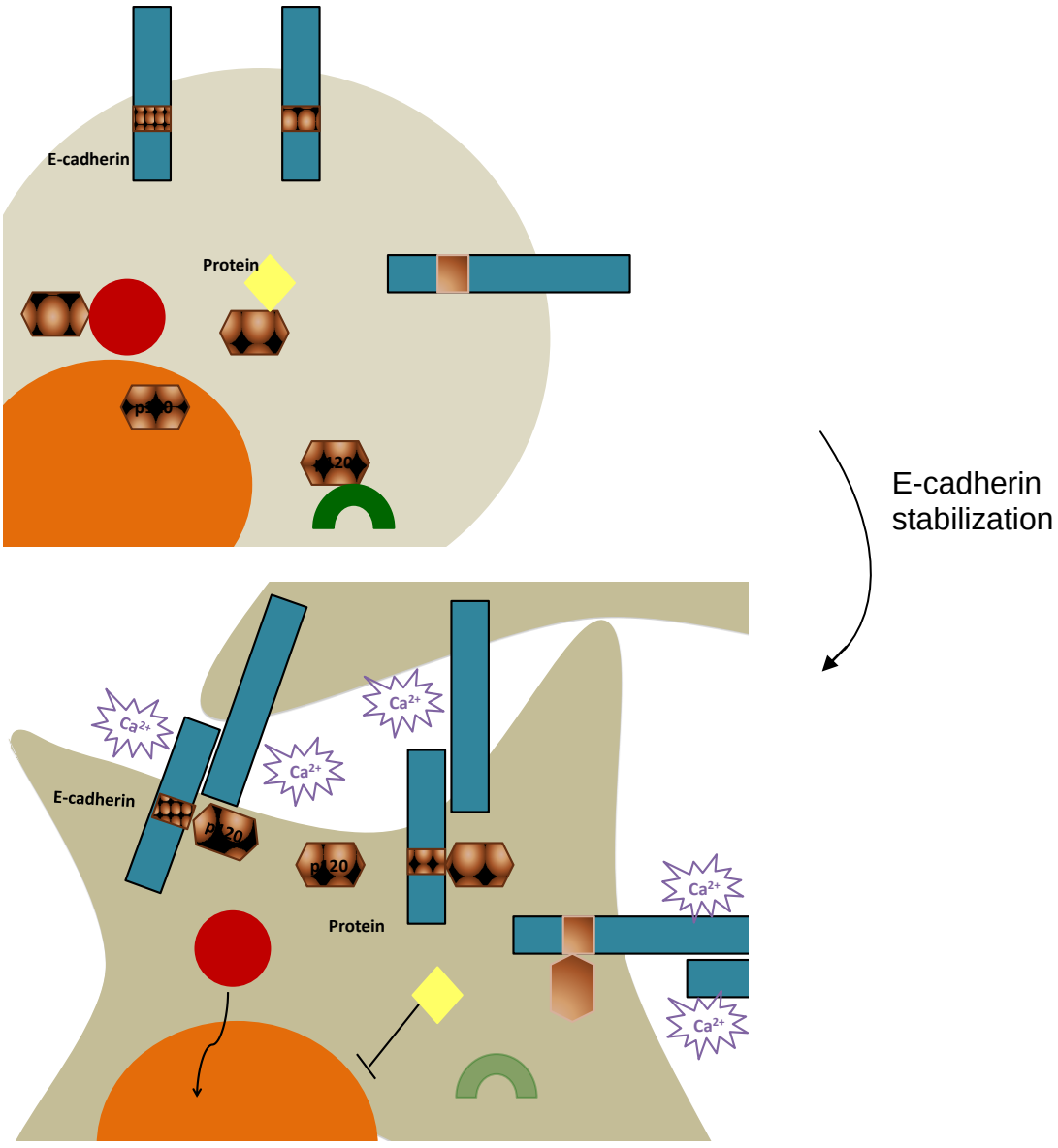


Diagram 1. Model of p120-catenin induced differentiation mechanism. p120-catenin sequestration to E-cadherin may deplete nuclear p120-catenin or leave other binding partners to activate, repress, or degrade leading to changes in transcriptional activity affecting cell cycle events.

Future work

A caveat to this study is that all conclusions were drawn from overexpression experiments. In order to make more conclusive remarks on E-cadherin's ability to induce epidermal stem cell differentiation, we need to address whether E-cadherin engagement is sufficient to recapitulate the findings in the study. I have been working towards answering this question.

E-cad:Fc protein, the extracellular domains of murine E-cadherin fused to the Fc region of human IgG, will be employed in follow up experiments. Many attempts to purify Ecad:Fc secreted into media by transformed HEK293 cells were attempted. However, in nearly every purification, I was unable to stabilize the E-cad:Fc protein and it degraded. Ultimately, E-cad:Fc was acquired through R&D systems.

Basal keratinocytes were seeded at low confluency and low calcium atop E-cad:Fc coated plates. This eliminates known factors to promote differentiation aside from E-cadherin engagement. Some qPCR analysis of endogenous gene expression known to be involved in proliferation and differentiation has been examined. Preliminary results show that E-cadherin engagement alone does in fact alter gene expressions supporting findings in this study. However, further analysis and replications are still underway.

MATERIALS and METHODS

Antibodies

Immunofluorescence: Anti-IL2Rα (Santa Cruz), anti- T7 polyclonal (I Chemicon, Temecula, CA), anti-T7 monoclonal (Novagen, USA), anti-E-cadherin (Takara Bio Inc.)

Western blot: anti-human IgG HRP (Santa Cruz)

Cell Culture

Primary mouse keratinocytes were cultured in E-media with 10% fetal calf serum (Hyclone, Logan, UT) and supplemented with 1:9 conditioned E-media at 37 °C in a humidified atmosphere containing 7% CO_2. E-media is 3:1 (v/v) Dulbecco's modified Eagle's medium (DMEM, Gibco Labs, Grand Island, NY) and Ham's F-12 nutrient mixture (Gibco) with final concentrations of the following supplements: 0.4 ug/ml hydrocortisone (Sigma, St. Louis, MO); 10^{-10} M cholera enterotoxin (ICN Biomedicals); 5 ug/ml insulin (Sigma); 5ug/ml transferring (Sigma); 5 ug/ml T_3 (3,3',5-triiodo-L-thryonine) (Sigma); 50 units/ml penicillin, 50 ug/ml streptomycin (Gibco), 36.5 mM sodium bicarbonate, and 3.25 mM L-glutamine (Gibco). Conditioned media was prepared by incubating 3T3J2 cells at confluency in E-media for 24 hours and thereafter collected. All media contained 50 units/ml of penicillin and 50 µg/ml of streptomycin (Gibco). Primary keratinocytes were passaged at 80% confluency by using 0.25% trypsin. Transient transfections of keratinoctyes were performed using *Trans*IT-LT1

Reagent (Mirus). 20,000 mouse primary keratinocytes were seeded into a 24-well plate transfected 4 hours later. DNA was prepared and volume brought up to 25 ul per sample with serum-free E-media. 1.5ul of *Trans*IT-LT1 Reagent was added and mixed in 23.5ul of serum-free E-media per sample and incubated for 5 minutes at room temperature. 25ul of transfection mixture was then added to DNA mixtures for 50 ul total volume per sample and incubated at room temperature for 25 min prior to transfection. A constant amount of 500ng total DNA was used for different transfections.

Dual-luciferase luminescence measurement

Luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. For assays in 24-well plates, 100µl Passive Lysis Buffer was used to lyse cells in each well. For all assays, 20µl lysate was transferred to a luminometer tube, mixed with 100µl Luciferase Assay Reagent II and Fluc activity was read on a luminometer (MGM Instruments, Hamden, Connecticut, United States). Rluc activity was subsequently read after mixing 100µl Stop & Glo Reagent into the cell lysate containing Luciferase Assay Reagent IIExperiments were done in triplicate and repeated at least three times.

Constructs

E-cadherin constructs were a gift from the Gumbiner lab.

p120-catenin construct and p120-cateninΔB construct were generated by PCR using KOD plus polymerase (Toyobo) from mouse cDNA as template. The specific primer pair used for amplification of full length p120-catenin: forward primer with an EcoRV linker sequence, 5′-AAAAGATATCATGGACGACTCAGAGGTGGA-3′, along with a reverse primer with a NotI linker sequence and T7 tag, 5′-AAAAGCGGCCGCCTAACCCATTTGCTGTCCACCAGTCATGCTAGCCATAATCTTCTGCATGGGGGAGT-3′. The specific primer pair used for amplification of p120-cateninΔNES: forward primer with an EcoRV linker sequence, 5′-AAAAGATATCATGGACGACTCAGAGGTGGA-3′, along with a reverse primer with a NotI linker sequence and T7 tag, 5′-AAAAGCGGCCGCCTAACCCATTTGCTGTCCACCAGTCATGCTAGCCATAATCTTCTGCATCAAGGGTG-3′. The PCR product was purified with the Gel Extraction Kit (Promega, Madison, Wisconsin, United States) and ligated into pCDNA3 (Invitrogen, Carlsbad, California, United States). The gene was verified by sequencing and digested with EcoRV and NotI.

Immunofluorescence Assay

Cells were fixed with 4% formaldehyde in PBS. Blocking buffer (1X PBS / 5% normal donkey serum (Sigma) / 0.3% Triton X-100) applied for 15 minutes. Cells were then incubated in primary antibody overnight at 4°C. 1x Dapi used to stain nucleus. Images were acquired on an Olympus Bx51 microscope with an

Olympus DP70 camera. Acquisitions were performed using a 40× 1.3 UPlan FL N objective (Olympus).

RNA extraction and cDNA synthesis

Samples were homogenized with 500ul of TriZol reagent (Invitrogen; Carlsbad, CA) within respective wells. The homogenate was transferred to a 1.7-ml tube and centrifuged at 12,000 rpm at 4°C for 10 min. Next, 250 µl of the supernatant containing RNA was transferred to a fresh 1.7-ml tube and incubated at room temperature for 5 min. By vigorous hand shaking, 100 µl of chloroform was mixed with the sample and incubated at room temperature for 3 min. The tube then was centrifuged at 12,000 rpm at 4°C for 15 min. The aqueous phase, which now contained the RNA, was transferred to a fresh 1.7-ml tube. To precipitate the RNA, 250 µl of isopropyl alcohol was added, and the sample was incubated at room temperature for 10 min. The sample was centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was discarded, and the RNA pellet was washed with 1ml 75% ethanol. The sample was centrifuged at 7,500 rpm at 4°C for 10 min. The supernatant was discarded, and the RNA pellet was dried at room temperature for 5 min. The pellet was redissolved in 50 µl of RNase free water and incubated at 58°C for 10 min. Total RNA quality and concentration then were measured using spectrophotometry. Using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules,CA), cDNA was synthesized by adding iScript reagents including 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase, and sufficient nuclease-free water to a reaction volume of 20 µl. The reaction was

incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min, and then stored at −20°C.

Expression and purification of Ecad:Fc protein

Transformed Hek293 cell line secreting Ecad:Fc protein were a gift from the Nelson Lab. Hek293 cells were grown at 37°C in a humidified 5% CO_2 incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. All media contained 50 units/ml of penicillin, 50 µg/ml of streptomycin (Gibco), 250 ng/ml fungizone, and 40 µg/mL G418 (Gibco). After cells were grown to 90%+ confluency, plates were washed with PBS and replaced with media prepared as before serum-free. After 3 day incubation, media is collected, filtered, and ammonium sulfate is added to 50% saturation with gentle stirring overnight at 4°C. To precipitate protein, solution is centrifuged at 5000 rpm for 30 min at 4°C. Supernatant is discarded and protein pellet resuspended in 10ml PBS and dialyzed in PBS overnight at 4°C with 3 changes of PBS. Dialysate is then transferred to 15ml conical tube and incubated with end-over turning overnight at 4°C with Protein G Sepharose 4 Fast Flow (GE Healthcare). Ecad:Fc protein is then eluted off a column using 0.2M glycine-HCl pH 2.5 and neutralized with Tris-HCl pH 9.5. Eluates are stored at 4°C.

Ecad:Fc coating and qPCR

Ecad:Fc protein (R&D systems) was reconstituted in sterile 1x PBS pH 7.3 with Ca^{2+} and Mg^{2+} to 100ug/ml. Ecad:Fc in PBS was coated in 48-well plate, volume

was brought up to 100ul total to cover the well. Plate was incubated overnight (12+ hours) at 4°C. Mouse epidermis was separated using dispase. Basal keratinocytes were then harvested, 150,000 were seeded atop coated wells in CnT-57 media (CellNTec, USA), and plate was incubated at 37 °C in a humidified atmosphere containing 7% CO_2 for 48 hours. RNA and cDNA synthesis was performed as above. qPCR executed using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA) according to manufacturer's protocol on a CFX96™ Real-Time PCR System (Bio-Rad, USA).

REFERENCES

Aho, S., L. Levansuo, O. Montonen, C. Kari, U. Rodeck and J. Uitto (2002). "Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells." J Cell Sci **115**(Pt 7): 1391-1402.

Anastasiadis, P. Z., S. Y. Moon, M. A. Thoreson, D. J. Mariner, H. C. Crawford, Y. Zheng and A. B. Reynolds (2000). "Inhibition of RhoA by p120 catenin." Nat Cell Biol **2**(9): 637-644.

Aono, S., S. Nakagawa, A. B. Reynolds and M. Takeichi (1999). "p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells." J Cell Biol **145**(3): 551-562.

Benjamin, J. M. and W. J. Nelson (2008). "Bench to bedside and back again: molecular mechanisms of alpha-catenin function and roles in tumorigenesis." Semin Cancer Biol **18**(1): 53-64.

Blanpain, C. and E. Fuchs (2006). "Epidermal stem cells of the skin." Annu Rev Cell Dev Biol **22**: 339-373.

Blanpain, C. and E. Fuchs (2009). "Epidermal homeostasis: a balancing act of stem cells in the skin." Nat Rev Mol Cell Biol **10**(3): 207-217.

Blanpain, C., V. Horsley and E. Fuchs (2007). "Epithelial stem cells: turning over new leaves." Cell **128**(3): 445-458.

Braga, V. M., L. M. Machesky, A. Hall and N. A. Hotchin (1997). "The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts." J Cell Biol **137**(6): 1421-1431.

Candi, E., R. Schmidt and G. Melino (2005). "The cornified envelope: a model of cell death in the skin." Nat Rev Mol Cell Biol **6**(4): 328-340.

Cavallaro, U. and G. Christofori (2004a). "Cell adhesion and signalling by cadherins and Ig-CAMs in cancer." Nat Rev Cancer **4**(2): 118-132.

Cavallaro, U. and G. Christofori (2004b). "Multitasking in tumor progression: signaling functions of cell adhesion molecules." Ann N Y Acad Sci **1014**: 58-66.

Cavallaro, U., B. Schaffhauser and G. Christofori (2002). "Cadherins and the tumour progression: is it all in a switch?" Cancer Lett **176**(2): 123-128.

Chen, Y. T., D. B. Stewart and W. J. Nelson (1999). "Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and

basal-lateral membrane targeting of E-cadherin in polarized MDCK cells." J Cell Biol **144**(4): 687-699.

Dale, B. A., K. A. Resing and J. D. Lonsdale-Eccles (1985). "Filaggrin: a keratin filament associated protein." Ann N Y Acad Sci **455**: 330-342.

Davis, M. A., R. C. Ireton and A. B. Reynolds (2003). "A core function for p120-catenin in cadherin turnover." J Cell Biol **163**(3): 525-534.

Davis, M. A. and A. B. Reynolds (2006). "Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland." Dev Cell **10**(1): 21-31.

Di Croce, L. and P. G. Pelicci (2003). "Tumour-associated hypermethylation: silencing E-cadherin expression enhances invasion and metastasis." Eur J Cancer **39**(4): 413-414.

Elias, P. M., P. Nau, K. Hanley, C. Cullander, D. Crumrine, G. Bench, E. Sideras-Haddad, T. Mauro, M. L. Williams and K. R. Feingold (1998). "Formation of the epidermal calcium gradient coincides with key milestones of barrier ontogenesis in the rodent." J Invest Dermatol **110**(4): 399-404.

Frixen, U. H., J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Lochner and W. Birchmeier (1991). "E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells." J Cell Biol **113**(1): 173-185.

Fuchs, E. (1995). "Keratins and the skin." Annu Rev Cell Dev Biol **11**: 123-153.

Fuchs, E. and H. Green (1980). "Changes in keratin gene expression during terminal differentiation of the keratinocyte." Cell **19**(4): 1033-1042.

Gottardi, C. J., E. Wong and B. M. Gumbiner (2001). "E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner." J Cell Biol **153**(5): 1049-1060.

Gumbiner, B., B. Stevenson and A. Grimaldi (1988). "The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex." J Cell Biol **107**(4): 1575-1587.

Gumbiner, B. M. (1993). "Proteins associated with the cytoplasmic surface of adhesion molecules." Neuron **11**(4): 551-564.

Gumbiner, B. M. (1996). "Cell adhesion: the molecular basis of tissue architecture and morphogenesis." Cell **84**(3): 345-357.

Gumbiner, B. M. (2000). "Regulation of cadherin adhesive activity." J Cell Biol **148**(3): 399-404.

Gumbiner, B. M. (2005). "Regulation of cadherin-mediated adhesion in morphogenesis." Nat Rev Mol Cell Biol **6**(8): 622-634.

Huber, A. H., D. B. Stewart, D. V. Laurents, W. J. Nelson and W. I. Weis (2001). "The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover." J Biol Chem **276**(15): 12301-12309.

Keirsebilck, A., S. Bonne, K. Staes, J. van Hengel, F. Nollet, A. Reynolds and F. van Roy (1998). "Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms." Genomics **50**(2): 129-146.

Kemler, R., M. Ozawa and M. Ringwald (1989). "Calcium-dependent cell adhesion molecules." Curr Opin Cell Biol **1**(5): 892-897.

Knudsen, K. A., A. P. Soler, K. R. Johnson and M. J. Wheelock (1995). "Interaction of alpha-actinin with the cadherin/catenin cell-cell adhesion complex via alpha-catenin." J Cell Biol **130**(1): 67-77.

Kobielak, A. and E. Fuchs (2004). "Alpha-catenin: at the junction of intercellular adhesion and actin dynamics." Nat Rev Mol Cell Biol **5**(8): 614-625.

Koster, M. I. and D. R. Roop (2007). "Mechanisms regulating epithelial stratification." Annu Rev Cell Dev Biol **23**: 93-113.

Lock, J. G. and J. L. Stow (2005). "Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin." Mol Biol Cell **16**(4): 1744-1755.

Margulis, A., W. Zhang, A. Alt-Holland, H. C. Crawford, N. E. Fusenig and J. A. Garlick (2005). "E-cadherin suppression accelerates squamous cell carcinoma progression in three-dimensional, human tissue constructs." Cancer Res **65**(5): 1783-1791.

Marthiens, V., I. Kazanis, L. Moss, K. Long and C. Ffrench-Constant (2010). "Adhesion molecules in the stem cell niche--more than just staying in shape?" J Cell Sci **123**(Pt 10): 1613-1622.

Menon, G. K., P. M. Elias, S. H. Lee and K. R. Feingold (1992). "Localization of calcium in murine epidermis following disruption and repair of the permeability barrier." Cell Tissue Res **270**(3): 503-512.

Menon, G. K., S. Grayson and P. M. Elias (1985). "Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry." J Invest Dermatol **84**(6): 508-512.

Mo, Y. Y. and A. B. Reynolds (1996). "Identification of murine p120 isoforms and heterogeneous expression of p120cas isoforms in human tumor cell lines." Cancer Res **56**(11): 2633-2640.

Moon, R. T., B. Bowerman, M. Boutros and N. Perrimon (2002). "The promise and perils of Wnt signaling through beta-catenin." Science **296**(5573): 1644-1646.

Omary, M. B., P. A. Coulombe and W. H. McLean (2004). "Intermediate filament proteins and their associated diseases." N Engl J Med **351**(20): 2087-2100.

Ozawa, M. and R. Kemler (1998). "The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity." J Cell Biol **142**(6): 1605-1613.

Pokutta, S., K. Herrenknecht, R. Kemler and J. Engel (1994). "Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding." Eur J Biochem **223**(3): 1019-1026.

Reynolds, A. B., J. Daniel, P. D. McCrea, M. J. Wheelock, J. Wu and Z. Zhang (1994). "Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes." Mol Cell Biol **14**(12): 8333-8342.

Reynolds, A. B., L. Herbert, J. L. Cleveland, S. T. Berg and J. R. Gaut (1992). "p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo." Oncogene **7**(12): 2439-2445.

Reynolds, A. B. and A. Roczniak-Ferguson (2004). "Emerging roles for p120-catenin in cell adhesion and cancer." Oncogene **23**(48): 7947-7956.

Rimm, D. L., E. R. Koslov, P. Kebriaei, C. D. Cianci and J. S. Morrow (1995). "Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex." Proc Natl Acad Sci U S A **92**(19): 8813-8817.

Ringwald, M., R. Schuh, D. Vestweber, H. Eistetter, F. Lottspeich, J. Engel, R. Dolz, F. Jahnig, J. Epplen, S. Mayer and et al. (1987). "The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of Ca2+-dependent cell adhesion." EMBO J **6**(12): 3647-3653.

Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, K. Miyazawa, N. Kitamura, K. R. Johnson, M. J. Wheelock, N. Matsuyoshi, M. Takeichi and et al. (1995). "Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes." J Cell Biol **128**(5): 949-957.

Smalley-Freed, W. G., A. Efimov, S. P. Short, P. Jia, Z. Zhao, M. K. Washington, S. Robine, R. J. Coffey and A. B. Reynolds (2011). "Adenoma Formation following Limited Ablation of p120-Catenin in the Mouse Intestine." PLoS One **6**(5): e19880.

Steven, A. C. and P. M. Steinert (1994). "Protein composition of cornified cell envelopes of epidermal keratinocytes." J Cell Sci **107 (Pt 2)**: 693-700.

Sun, T. T., R. Eichner, W. G. Nelson, S. C. Tseng, R. A. Weiss, M. Jarvinen and J. Woodcock-Mitchell (1983). "Keratin classes: molecular markers for different types of epithelial differentiation." J Invest Dermatol **81**(1 Suppl): 109s-115s.

Takaishi, K., T. Sasaki, H. Kotani, H. Nishioka and Y. Takai (1997). "Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells." J Cell Biol **139**(4): 1047-1059.

Takeichi, M. (1991). "Cadherin cell adhesion receptors as a morphogenetic regulator." Science **251**(5000): 1451-1455.

Takeichi, M. (1995). "Morphogenetic roles of classic cadherins." Curr Opin Cell Biol **7**(5): 619-627.

Timpl, R., H. Rohde, P. G. Robey, S. I. Rennard, J. M. Foidart and G. R. Martin (1979). "Laminin--a glycoprotein from basement membranes." J Biol Chem **254**(19): 9933-9937.

Tinkle, C. L., T. Lechler, H. A. Pasolli and E. Fuchs (2004). "Conditional targeting of E-cadherin in skin: insights into hyperproliferative and degenerative responses." Proc Natl Acad Sci U S A **101**(2): 552-557.

van Hengel, J. and F. van Roy (2007). "Diverse functions of p120ctn in tumors." Biochim Biophys Acta **1773**(1): 78-88.

van Hengel, J., P. Vanhoenacker, K. Staes and F. van Roy (1999). "Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression." Proc Natl Acad Sci U S A **96**(14): 7980-7985.

Vleminckx, K., L. Vakaet, Jr., M. Mareel, W. Fiers and F. van Roy (1991). "Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role." Cell **66**(1): 107-119.

Watabe-Uchida, M., N. Uchida, Y. Imamura, A. Nagafuchi, K. Fujimoto, T. Uemura, S. Vermeulen, F. van Roy, E. D. Adamson and M. Takeichi (1998). "alpha-Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells." J Cell Biol **142**(3): 847-857.

Xiao, K., J. Garner, K. M. Buckley, P. A. Vincent, C. M. Chiasson, E. Dejana, V. Faundez and A. P. Kowalczyk (2005). "p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin." Mol Biol Cell **16**(11): 5141-5151.

Yagi, T. and M. Takeichi (2000). "Cadherin superfamily genes: functions, genomic organization, and neurologic diversity." Genes Dev **14**(10): 1169-1180.

Yanagisawa, M., D. Huveldt, P. Kreinest, C. M. Lohse, J. C. Cheville, A. S. Parker, J. A. Copland and P. Z. Anastasiadis (2008). "A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease." J Biol Chem **283**(26): 18344-18354.

Yap, A. S. (1998). "The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review." Cancer Invest **16**(4): 252-261.

Yap, A. S., W. M. Brieher, M. Pruschy and B. M. Gumbiner (1997). "Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function." Curr Biol **7**(5): 308-315.

Yap, A. S., C. M. Niessen and B. M. Gumbiner (1998). "The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn." J Cell Biol **141**(3): 779-789.

Young, P., O. Boussadia, H. Halfter, R. Grose, P. Berger, D. P. Leone, H. Robenek, P. Charnay, R. Kemler and U. Suter (2003). "E-cadherin controls adherens junctions in the epidermis and the renewal of hair follicles." EMBO J **22**(21): 5723-5733.

Yuspa, S. H., A. E. Kilkenny, P. M. Steinert and D. R. Roop (1989). "Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro." J Cell Biol **109**(3): 1207-1217.

Zhang, J., J. J. O'Donnell, 3rd, O. Holian, P. A. Vincent, K. S. Kim and H. Lum (2010). "P120 catenin represses transcriptional activity through Kaiso in endothelial cells." Microvasc Res **80**(2): 233-239.