ON THE CONTRIBUTION OF SELF-MEDIATED ORGANIZATION OF KERATIN
INTERMEDIATE FILAMENTS TO THEIR BIOLOGICAL FUNCTION *IN VIVO*

by
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ABSTRACT

The crucial role of structural support fulfilled by keratin intermediate filaments (IFs) in surface epithelia likely requires that they be organized into crosslinked networks. For IFs comprised of keratins 5 and 14, found in basal epidermal keratinocytes, formation of crosslinked bundles is, in part, self-driven through cis-acting determinants. Here, we targeted the expression of a bundling-competent *KRT5/KRT8* chimeric cDNA (*KRT8bc*), or bundling-deficient wildtype *KRT8* as a control, to the epidermal basal layer of *Krt5* null mice to assess the functional importance of keratin IF self-organization *in vivo*. We report that targeted expression of K8bc rescued *Krt5* null mice with ~47% frequency, while K8 failed to rescue. This outcome correlated with lower than expected levels of K8bc and especially K8 mRNA and protein in the epidermis of E18.5 replacement embryos. Electron microscopy of E18.5 embryonic skin revealed that the defects observed in filament bundling, cytoarchitecture, and mitochondria are partially restored by *KRT8bc* expression. As young adults, viable *KRT8bc* replacement mice develop alopecia and chronic skin lesions, indicating that the skin epithelia are not completely normal. These findings are consistent with a contribution of self-mediated organization of keratin IFs to structural support and cytoarchitecture in basal layer keratinocytes of the epidermis, and underscore the importance of context-dependent regulation for keratins *in vivo*.

Structural support is not the only function fulfilled by K5 in skin. A recent genome-wide association study has identified novel single nucleotide polymorphisms (SNPs) altering the K5 coding sequence and conferring an increased susceptibility to

basal cell carcinoma (BCC). We conducted an initial assessment of the properties of the

K5 variants G138E and D197E in cultured HeLa cells. We found that these two keratin

variants readily integrate into endogenous filament networks of HeLa cells, and do not

exhibit any egregious assembly properties. We also show that these mutants do not alter

the expression of early effectors in Sonic hedgehog (Shh) or Wnt signaling pathways.

Relative to wildtype, expression of these *KRT5* variants alter the RNA expression of two

inflammatory cytokines, *CCL2* and *CXCL5*. These findings point to a possible

mechanism by which *KRT5* SNPs may increase BCC susceptibility.

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CHAPTER 1

INTRODUCTION

The Keratin Gene Family

The most basic form of life on the planet is the cell, and all cells have some form

of a cytoskeletal network. In metazoans, this cytoskeleton consists of three fibrous

polymers: microfilaments, intermediate filaments, and microtubules. Together, these

cytoskeletal proteins fulfill many functions in the cell, including maintenance and

modification to cell shape, the ability to form cellular structures and appendages, cell

division, locomotion within their environment, and the ability to fend off stress.

Intermediate filaments can be categorized into a variety of subtypes according to

their gene structure and sequence homology (Fig. 1.1). Intermediate filaments (IFs)

were first described in muscle by Holtzer and colleagues as 10nm-wide filamentous

elements, thus distinct from and intermediate in diameter relative to the already known

actin filaments (6nm) and microtubules (23nm) (Ishikawa et al., 1968). Although IF-

forming cytoskeletal proteins are quite diverse, they all share a similar tripartite domain

structure. The hallmark of this structure is a central rod domain, which is 310 amino

acids long in virtually all IF proteins and consist of four α-helical subdomains separated

by three short non-helical linker segments (Fig 1.1). This central rod domain is flanked

by a head domain and a tail domain at the amino- and carboxy-terminal ends of the rod

domain, respectively (Steinert and Parry, 1985). These head and tail domains vary

greatly between members of this superfamily in both length and primary structure.

Keratins make up the majority of this protein superfamily, accounting for 54 of the 70

functional IF genes in humans (Chung et al., 2013). Keratin genes can be further divided

Figure 1.1: Introduction to Intermediate Filaments (IFs). A) Classification of IF genes and proteins by type, according to gene substructure and sequence homology, and cell type-specificity of their distribution in the body (note: the latter list is partial). B) Visualization of assembled 10nm-wide IFs reconstituted from purified recombinant proteins (the type II K5 and type I K14; human) by negative staining and transmission electron microscopy. Bar equals 100nm. C) Schematic representation of the common tripartite domain structure shared by all IF proteins. A central rod domain, comprised of heptad repeat-containing α-helical coils 1A, 1B, 2A, and 2B and separated by non-heptad repeat-containing linkers L1, L12, and L2, is flanked by 'head' and 'tail' domains of variable length and primary structure at the N-termini and C-termini, respectively. The boundaries of the rod domain (see blue bars) are highly conserved in primary structure among IF proteins. Image reproduced with permission from Chung et al., 2013.

Figure 1.1: Introduction to Intermediate Filaments (IFs).

(a)

Subgrouping	Proteins	Cell type specificity
Type I	Keratins	Soft complex epithelia (skin, oral mucosa, etc.)
Type II		Soft simple epithelia (liver, gut, kidney, etc.)
		Hard epithelia (hair, nail, oral papillae)
Type III	Vimentin, Desmin	Various (fibroblasts, leukocytes, endothelium
	GFAP, Peripherin	muscle, astrocytes, glia, peripheral nerves)
	syncoilin	
Type IV	NF-L, NF-M, NF-H	CNS & neurons
	a-internexin	CNS & neurons
	synemin, nestin	Muscle, neural stem cells
Type V	Lamins A, B & C	Nucleus
Orphan	Filensin, Phakinin	Lens

(b)

(c)
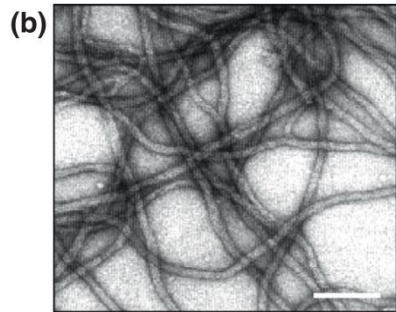
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into two types: type I, of which there are 28 functional genes encoding smaller sized proteins (range: 40-64 kilodaltons [kDa]) with an acidic isoelectric point, and type II keratins, of which there are 26 genes encoding proteins of a slightly large size (52-67 kDa) and a basic to neutral isoelectric point (Moll et al., 1982).

Assembly of 10nm filaments is initiated by the association of one type I keratin and one type II keratin associate along their central α-helical rod domain, in register and parallel orientation, to form a structurally asymmetric coiled-coil dimer (Herrmann and Aebi, 2004). Two of these type I/ type II keratin heterodimers then associate with each other, in a staggered and antiparallel fashion, resulting in structurally symmetric heterotetramers (Herrmann and Aebi, 2004; Bernot et al., 2005). These heterotetramers interact with each other, end-to-end and along their lateral surfaces, to give rise to mature 10nm filaments (Herrmann and Aebi, 2004; Kim and Coulombe, 2007; Lee et al., 2012). On average, mature 10nm filaments are comprised of 32 polypeptide chains across their width, which are organized into four so-called protofibrils intertwined along the main axis of the fiber (Herrmann and Aebi, 2004; Kim and Coulombe, 2007; Lee et al., 2012). The resulting filaments interact with each other, with cell-cell and cell-matrix junctions, and with the nuclear surface to form a classic pan-cytoplasmic network (Fuchs and Cleveland, 1998; Coulombe and Wong, 2004; Wilhelmsen et al., 2005).

Organization and Regulation of Keratin Gene Expression

All functional type I and type II keratin genes are clustered on the long arms of chromosomes 17 and 12, respectively, in the human genome, an arrangement that is

conserved in other mammalian genomes (Hesse et al., 2004). The only exception is the gene encoding the type I keratin 18, which is located at the telomeric end of the type II cluster (Waseem et al., 1990). This peculiar organization has implications for the evolution and regulation of keratin genes (Waseem et al., 1990), but is poorly understood at present.

Because filament assembly is initiated with one of each type of keratin proteins, the regulation of keratin genes is tightly regulated in a pairwise fashion, with type I and type II proteins being expressed in specific epithelial compartments (Moll et al., 1982; Tyner and Fuchs, 1986). For example, cells in the *stratum basale* (basal layer) of skin epidermis express the type II keratin 5 (K5) and the type I keratins 14 and 15 (K14 and K15) (Fuchs and Green, 1980; Nelson and Sun, 1983; Lloyd et al., 1995). Upon commitment to terminal differentiation program, the expression of these keratins is down regulated at the transcriptional level, and the expression of the type I keratin K10 and the type II keratin K1 is initiated (Fuchs and Green, 1980; Woodcock-Mitchell et al., 1982). This shift in keratin expression occurs concomitantly with the cell's exit from the basal compartment and from the cell cycle, and its entry into the *stratum spinosum* (spinous layer) of the epidermis. Eventually, expression of the type II keratin K2e is initiated in the *stratum granulosum* (granular layer), which corresponds to a more advanced stage of terminal differentiation (Collin et al., 1992; Fuchs, 1995). Finally, cells complete their differentiation program and become metabolically-inactive squames, which make up the *stratum corneum* (cornified layers) of the mature epidermis (Fuchs and Green, 1980). In contrast, simple epithelia, e.g., the liver, intestinal tract, and pancreas, primarily express

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the type II keratins K7/K8 and type I keratins K18, K19, and K20 in combinations that are often specific to the tissue (Ku et al., 1999). For example, hepatocytes express K8 and K18 exclusively, while the epithelial cells which line the colon also express K19 and K20 (Ku et al., 1999).

The mechanism behind this tight, context-specific regulation of keratin genes is an area of active research. Work in the context of skin epithelia has revealed a unique set of binding sites in the promoters of keratins, which overlap in keratin pairs that are expressed in specific contexts (Leask et al., 1991; Byrne et al., 1994; Sinha et al., 2000). In the case of human *KRT5* and *KRT14* genes, expression is restricted to the basal cell layer in the epidermis by interaction of a combination of transcription factors with regions located upstream of the coding region of the genes (Leask et al., 1991; Byrne et al., 1994). Transcription factors such as AP-2 and Sp1 have been shown to be positive regulators of the expression of these genes in basal cells (Byrne, 1997). Conversely, POU domain proteins have been shown to negatively regulate the expression of these genes in the suprabasal layers of the epidermis (Andersen et al., 1997; Faus et al., 1994). Other transcription factors and regulators, including NF-κB and Notch, have also been shown to be involved in this complicated regulation paradigm (Seitz et al., 1998; Wang et al., 2008).

Keratins are also regulated at the protein level through several types of post-translational modifications. For example, phosphorylation of keratins was first described in 1978, and has since been shown to play an important role in regulating keratins (Sun and Green, 1978; Omary et al., 2006; Snider and Omary, 2014). The importance of this

modification is reflected by the presence of numerous potential serine/threonine

phosphorylation sites in their non-helical head and tail domains (Omary et al., 2006;

Izawa and Inagaki, 2006; Snider and Omary, 2014). The effect of this post-translational

modification is, like the keratin proteins themselves, context- and protein- specific (Pan et

al., 2013; Snider and Omary, 2014). Since keratin filaments are stable structures within

the cell, one mode of action of phosphorylation is to regulate the solubility of keratin

assembly subunits (Nishizawa et al., 1991; Yano et al., 1991; Izawa and Inagaki, 2006).

For instance, cells undergoing mitosis often reorganize their IF network, and this is

achieved in part through site-specific phosphorylation (Nishizawa et al., 1991; Yano et

al., 1991; Toivola et al., 2002; Woll et al., 2007). Phosphorylation-based regulation of

IFs also takes place in cells responding to stress or undergoing apoptosis (Toivola et al.,

2002; Ridge et al., 2005; Liao et al., 1997). It is clear from these studies that

phosphorylation of keratin proteins on residues located in the C-terminal tail and

especially the N-terminal head domains induces a reorganization of the filament structure

(Omary et al., 2006; Snider and Omary, 2014).

Keratin filaments tend to be long-lasting structures in the epithelial cells in which

they occur (Omary et al., 2006). They can be, however, very dynamic depending on the

biological context. While phosphorylation of keratin proteins or their interaction with

cellular proteins (e.g., 14-3-3 proteins) play a role in the reversible reorganization or

disassembly of filaments into their constituent tetramers, the ubiquitin-proteasome

pathway is involved in the irreversible degradation of their constituent proteins (Rogel et

al., 2010). In this pathway, ubiquitin is covalently attached to target proteins via a three-

enzyme cascade (Ciechanover and Schwartz, 1998). The first two steps of this cascade, the activation of the ubiquitin molecule by E1 and the transfer of the ubiquitin to a conjugating enzyme by E2, are generally conserved among the many targets within a cell (Ciechanover and Schwartz, 1998). The final step of covalently linking ubiquitin to target proteins is carried out by E3 ubiquitin ligases, which are unique to the proteins targeted (Ciechanover and Schwartz, 1998). A number of stress-related E2 enzymes have been implicated in keratin ubiquitylation, including UbcH5b, UbcH5c, Ubc3, and Ubc6 (Jaitovich et al., 2008; Na et al., 2010; Rogel et al., 2010). While the E3 ligases specific to wildtype keratins are still unknown, the chaperone-associated E3 ligase CHIP/STUB1 has been shown to target mutant keratins for degradation (Loffek et al., 2010). This is also relevant in the context of human diseases, as both wildtype and mutant keratin proteins often form cellular aggregates in keratinopathies (Ku et al., 1996; Jaitovich et al., 2008; Rogel et al., 2010; Loffek et al., 2010).

Other post-translational modifications have also been shown to impact keratin regulation and dynamics. For instance, O-linked N-acetylglucosamine glycosylation and SUMOylation have both been shown to regulate filament organization (Srikanth et al., 2010; Snider et al., 2011). Modification of keratin proteins with these and other adducts alters the protein surfaces available for protein-protein interactions. As such, the interactome of modified keratin proteins is likely different from that of unmodified keratins (Pan et al., 2013). For example, in the context of cellular stress, keratins are ubiquitylated in a phosphorylation-dependent fashion, indicating a bridge between these two post-translational modification pathways (Ku and Omary, 2000; Kwan et al., 2012).

Elucidating the roles of these post-translational modifications and differentially-interacting proteins is an area of emerging discoveries. Another fruitful field of investigation is the role of these post-translational modifications in non-mechanical functions distinct from filament reorganization. These non-mechanical functions will be discussed in a later section of this dissertation.

Keratin Intermediate Filaments Impart Structural Support to Epithelia

The complex and context-specific regulation of keratin genes and proteins reflect their important functions in epithelia. Clues of these functions were first uncovered by expressing cDNAs of keratin 14 deletion mutants in cells (Albers and Fuchs, 1987; Albers and Fuchs, 1989; Coulombe et al., 1990). The protein products of these cDNAs were shown to have a dominant and negative effect on the existing filament network in cells (Albers and Fuchs, 1987; Albers and Fuchs, 1989; Coulombe et al., 1990). It was also determined that deletions in the amino terminal rod domain lead to filament assembly disruption (Coulombe et al., 1990). A major breakthrough in skin biology occurred when these keratin 14 deletion mutant cDNAs were expressed in the epidermis of mice (Vassar et al., 1991; Coulombe et al., 1991b). Mice expressing these deletion mutants presented with basal cell fragility, a phenotype which was similar to the human genetic disease Epidermolysis Bullosa simplex (EBS) (Vassar et al., 1991; Coulombe et al., 1991b). The hallmark of this genetic disorder is cell and tissue fragility, and as such patients with this disease present with bullous lesions on sites of frictional trauma (Coulombe and Fuchs, 1993; Coulombe et al., 2009). The reason for the similarities between the mouse models and EBS patients became clear when keratin 14 was cloned

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from EBS patients and mutations in the rod domain were identified (Coulombe et al., 1991a). The behavior of these keratin mutants were observed *in vitro* and in transfected cells, and suggested a causal relationship between keratin mutation and filament network disruption (Coulombe et al., 1991a). In this setting, it was observed that the filament assembly was disrupted in these variants, and that cells expressing these variants did not form the pan-cytoplasmic networks observed with wildtype keratins (Coulombe et al., 1991a). These data became the first of many pieces of evidence that keratins provide structural support to the tissues in which they are expressed (Coulombe et al., 1991b; Coulombe et al., 2009).Further evidence in support of this function for keratin intermediate filaments was provided by the genetic ablation of *Krt14* (Lloyd et al., 1995) in mice. In this model, the animals presented with severe tissue fragility, resulting in the formation of large blisters over most of the neonates' bodies (Lloyd et al., 1995). It was further shown that the blisters were formed as a result of cytolysis of the basal cells of the epidermis, with the cytolysis occurring through the plane basal cells, much like what occurs in earlier mouse models and EBS patients (Vassar et al., 1991; Coulombe et al., 1991a; Coulombe et al., 1991b; Lloyd et al., 1995). The majority of these *Krt14* knockout mice died two days after being born; however, due to the expression of another type I keratin in the basal cells, K15, some animals survived to adulthood (Lloyd et al., 1995).

Later, the function of keratin filaments as structural support networks was again highlighted in the genetic ablation of a keratin gene, this time of K14's binding partner in the basal epidermis, *Krt5* (Peters et al., 2001). However, this mouse model displayed a

more severe phenotype than that observed in the *Krt14* null mice. These animals experienced severe tissue fragility at the time of birth, and all pups died within an hour of birth (Peters et al., 2001). This phenotype supported the function assigned to keratin filaments by previous work. The fact that the phenotype of the *Krt5* null mice was more severe than that observed in the *Krt14* null mice is likely due to the expression of another type I keratin in the basal cells of the epidermis, keratin 15 (Lloyd et al., 1995). Keratin 5 is the only type II keratin expressed in these cells, and therefore the consequences of the loss of this binding partner was reflected in the increased severity of the mouse phenotype (Peters et al., 2001).

EBS is not the only human disease that provides insight on the function of keratin filaments. A similar disorder, called epidermolytic hyperkeratosis (EHK), was shown to also be caused by mutations in keratins 1 and 10, which are expressed in the *stratum spinosum* (Fuchs et al., 1992; Cheng et al., 1992; Chipev et al., 1992). Another disorder called epidermolytic palmoplantar keratoderma (EPPK), which is hallmarked by blisters occurring in the palms and soles of patients, has been shown to be caused by mutations in keratin 9, a type I keratin specifically expressed in this type of epidermis (Reis et al., 1994). Also, genetic ablation of keratin 6 leads to massive blistering secondary to the acute fragility of filliform papillae in the dorsal aspect of the tongue (Wong et al., 2000). Disorders such as EBS, EHK, EPPK, and others are caused by mutations in keratins that are expressed under normal conditions in these tissues; however, tissue fragility is not limited to mutations in keratins expressed in intact epithelia. For example, keratin 6, along with keratins 16, and 17 are induced at the wound edge within hours of injury

(Paladini et al., 1996). Hence, the fragility of *Krt6* null keratinocytes is also observable at the wound edge of skin grafts from these animals (Wong and Coulombe, 2003).

Further understanding of the structural support function of keratin filaments in mice was yielded through a number of protein replacement experiments. The basis of these experiments has been to generate a mouse which is null at a given keratin gene locus, and is engineered to express an alternative keratin by means of a transgene. A great deal of information has been garnered from experiments like this. For instance, expression of the simple keratin K18 could not rescue the tissue fragility incurred due to the loss of keratin 14 (Hutton et al., 1998), revealing that keratin filaments possess unique properties dependent on their protein makeup. Other replacement experiments revealed there is a surprising level of functional diversity between keratins. For example, the same study showed that a chimeric keratin with the head and rod domains of keratin 16 and the tail domain of keratin 14 was sufficient to rescue the blistering phenotype in the *Krt14* null mice (Hutton et al., 1998). In contrast, expression of wildtype human keratin 16 under the direction of the keratin 14 promoter in the epidermis of transgenic mice results in hyperproliferation of these cells, leading to hyperkeratosis of the tissue (Paladini and Coulombe, 1998). When these animals were used in a breeding strategy for replacement of keratin 14, the initial phenotype subsided to some degree, but the stratified epithelia of the resulting replacement mice were not completely normal (Paladini and Coulombe, 1999).

Another experiment highlighted the likelihood of functional redundancy among specific subgroups of highly homologous keratin proteins. For instance, when keratin 17

was ectopically expressed in the basal cells of *Krt14* null mouse epidermis by either the expression of a *GLI2* transgene or by treatment with a chemical activator of Nrf2, this correlated with a significant rescue of the skin fragility phenotype of *Krt14* null mice (Kerns et al., 2007). This finding also provided further evidence for the induction of these wound-inducible keratins as the reason most sites of blistering in human EBS patients heal without scarring (Coulombe et al., 1991b; El Ghalbzouri et al., 2003; Kerns et al., 2007). In each of these cases, the message was clear: cells and tissues require the structural support function provided by intact keratin filaments (Coulombe et al., 2009).

Organization of Keratin Filaments into Cross-Linked Networks

A common theme regarding structural support among the classes of cytoskeletal proteins is the formation of cross-linked networks to increase mechanical resilience (Janmey et al., 1991; Coulombe et al., 2000). For intermediate filaments, one way this is achieved is by the formation of stable non-covalent interactions within filaments as well as with interacting proteins (Fuchs and Cleveland, 1998; Yamada et al., 2002; Lee and Coulombe, 2009). At sites of cell-cell and cell-matrix anchorages, keratin filaments interact with members of the plakin family, such as desmoplakin and plectin, which serve as molecular bridges between cytoplasmic intermediate filaments and the junctional complexes of desmosomes and hemidesmosomes, respectively (Fuchs and Cleveland, 1998; Coulombe and Wong, 2004; Sonnenberg and Liem, 2007). Keratin intermediate filaments are also anchored at the surface of the nucleus via interaction with plectin, and plectin's subsequent interaction with nesprin-3 (Wilhelmsen et al., 2005). These interactions complement one another towards forming a mechanically-sound intra- and

supra-cellular network able to withstand deformation (Fuchs and Cleveland, 1998; Yamada et al., 2002).

Keratin filaments are also subject to modification by several cross-linking enzymes and interacting proteins. At a very late stage of terminal differentiation in the epidermis, for example, keratins are acted upon by transglutaminases and cross-linked by filaggrin, loricrin, and trichohyalin as the cornified envelope forms and matures (Steinert and Marekov, 1995; Lee et al., 1993). For epithelial appendages such as hair, keratins participate in extensive cross-linking through the hair keratin proteins' cysteine-rich non-helical regions (Fuchs and Cleveland, 1998). In both of these examples, cross-linking keratin filaments results in a rigidly bundled structure, enhancing mechanical resilience and aiding in the genesis of chemical and physical barriers.

Despite the wealth of knowledge pertaining to keratin cross-linking, the mechanisms of keratin cross-linking in the general cytoplasm of basal progenitor cells remain unclear. A possible explanation of this was identified during a study of the role of keratin protein domain requirements during filament assembly (Wilson et al., 1992). This study demonstrated that alterations in the assembly buffer conditions and of the keratin protein structure via mutagenesis changed the propensity of the filaments to form large bundles *in vitro* (Wilson et al., 1992). This observation was later determined to be intrinsic to the keratin makeup of the filaments, and correlated with a markedly increased elasticity (i.e., mechanical resilience) of filament suspensions (Ma et al., 2001; Bousquet et al., 2001; Yamada et al., 2002). The pursuit of such efforts eventually determined that a cross-linked organization of keratin filaments could be promoted *in vitro* depending on

the ionic strength and/or the pH of the assembly buffer (Ma et al., 2001; Yamada et al., 2002). This property of self-mediated organization into bundles was established as the intrinsic pathway of keratin filament cross-linking (Coulombe et al., 2000; Lee and Coulombe, 2009). Follow-up studies performed with K5 and K14 identified specific domains within each protein which were responsible for this phenomenon (Lee and Coulombe, 2009). Specifically, the distal portion of the tail domain in keratin 14 and two regions within the rod domain of keratin 5 play key roles in conferring this property, both *in vitro* and in transfected cells in culture (Lee and Coulombe, 2009). Such findings, along with the absence of cytoplasmically-localized filament cross-linkers in basal keratinocytes of the epidermis, suggest an important role for the so-called intrinsic pathway of keratin filament bundling in their structural support function *in vivo* (Fig 1.2). Initial efforts to explore this role in animal models are the subject of Chapter 2 of this dissertation.

Keratins as Modulators of Cytoarchitecture

Since keratins make up a significant percentage of cellular protein content (Sun and Green, 1978; Feng et al., 2013), they are poised to impact a large number of cellular processes (Kim and Coulombe, 2007; Pan et al., 2013). Moreover, the pan-cytoplasmic distribution of keratin filaments, especially in surface epithelia, allows them to play significant roles in cytoarchitecture. A recent study demonstrated that organization of keratin filaments into cross-linked networks significantly alters the rate of spreading and surface area of skin keratinocytes in culture, likely owing to generation of inward tension (Lee and Coulombe, 2009).

Figure 1.2: Mechanisms of Keratin Filament Bundling in Basal Keratinocytes of Epidermis. Ultrastructural examination shows that keratin filaments are abundant and show a loosely bundled organization in basal layer keratinocytes of epidermis. Although the molecular basis for the attachment of keratin filaments at cell–cell and cell–matrix adhesion sites is well understood, the mechanisms responsible for filament bundling in the general cytoplasm are unknown. We propose that in basal keratinocytes and related cell types, this organization results from contributions from both intrinsic (e.g., self-driven) and extrinsic (e.g., associated proteins such as plectin and epiplakin) determinants. Both determinants are postulated to be required for the key structural support role fulfilled by K5/K14 filaments in basal keratinocytes. Self-organization of K5/K14 filaments into cross-linked networks involves interaction between the short T2 segment at the extreme C terminus of K14, and two distinct regions in K5 (the "head-1A" and "L2-2B" regions) contributed by adjacent filaments within a bundle. Exposure of the K14 tail domain at the filament surface is directly supported by experiments that involved partial proteolysis of assembled K5/K14 filaments. The two regions in K5 rod domain are both required for full expression of the self-organization potential. The "head-1A" and "L2-2B" regions are ~300Å apart in K5, implying that the small K14's T2 segment (25 residues long) is too short to engage both of them within the same K5 molecule. By virtue of its richness in threonine and especially serine residues, K14's T1 domain is poised to regulate T2 function, and thus the intrinsic pathway of K5/K14 filament organization, via phosphorylation. Image reproduced with permission from Lee and Coulombe, 2009.

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Figure 1.2: Mechanisms of Keratin Filament Bundling in Basal Keratinocytes of Epidermis.
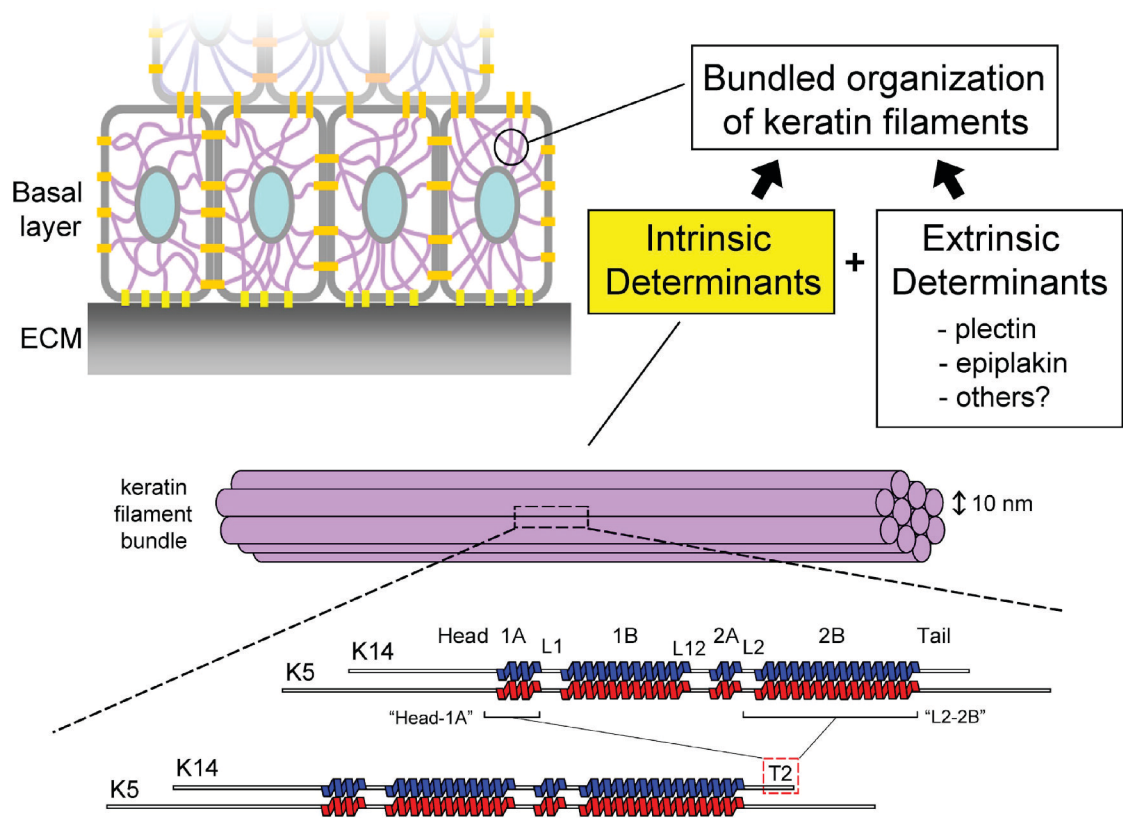
Keratin filaments have also been shown to alter the shape, and in turn the function, of specific organelles within the cell, such as mitochondria (Stone et al., 2007; Kumemura et al., 2008; Tao et al., 2009). Interestingly, the effect of keratins on mitochondria is dependent on the specific keratin protein and tissue being considered. In the case of keratin 19 null mice, skeletal muscle mitochondria are larger and show significant reorganization (Stone et al., 2007). On the other hand, mitochondria are significantly smaller in the liver of keratin 8 null animals, and their hepatocytes are more susceptible to apoptosis (Tao et al., 2009). This is also the case in mice expressing mutant keratin 18 (Kumemura et al., 2008). Keratin filaments are also tethered to another organelle, the nucleus, through interactions involving plectin and nesprin-3 (Wilhelmsen et al., 2005). More recently, a trans-dimer, homotypic disulfide bond involving residue Cysteine 367 in K14 was shown to promote the formation and/or maintenance of a cage of keratin filaments around the nucleus, impacting its size and shape in early differentiating epidermal keratinocytes (Lee et al., 2012). Such an impact on the nucleus by keratin filaments suggests a potential role in regulating differentiation, as well as impacting other functions of the nucleus (Lee et al., 2012; Pan et al., 2013).

Keratins Protect Against Cellular Stress

Keratins 8, 18, and 19 are normally expressed in the simple epithelia of the digestive system, including the liver (Ku et al., 1999). In this setting, the formation of intracellular aggregates known as Mallory-Denk bodies is an indicator of disease (Zatloukal et al., 2007; Strnad et al., 2012). Keratins are the major components of these aggregates, and are hyperphosphorylated and cross-linked (via transglutaminases) within

these aggregates (Strnad et al., 2012). It has been demonstrated that the formation of these aggregates is modulated by altering the ratio of K8 to K18 in hepatocytes, with higher keratin 8 expression predisposing toward the formation of Mallory-Denk bodies (Zatloukal et al., 2007; Strnad et al., 2012). Post-translational modification of these simple epithelial keratins also play significant roles in the maintenance of the organs in which they are expressed (Ku and Omary, 2006; Rotty et al., 2010). For instance, the phosphorylation of keratin 8 on serine 73 was shown to be necessary to protect the liver from Fas-mediated apoptosis (Ku and Omary, 2006). Additionally, transgenic mice expressing a mutant keratin 18 incapable of being glycosylated appear phenotypically normal, but when metabolically challenged present with increased apoptosis (Ku et al., 2010). These studies demonstrate that simple epithelial keratins act as cytoprotective entities against a variety of insults in the liver (Ku et al., 2007).

Role of Keratins in Apoptosis

Exploration into the mechanisms behind the observed cytoprotection in simple epithelia has revealed that keratins are involved in a number of signaling cascades within the cell. In this setting, keratins 8 and 18 can protect against TNFα-induced apoptosis through the binding and sequestration of TRADD, a downstream signaling molecule in the TNFα-induced apoptotic pathway (Inada et al., 2001). Mice lacking keratin 8 have an increased susceptibility to apoptosis via this pathway (Inada et al., 2001). The interaction between keratins and TRADD does not seem to be limited to simple epithelia. Keratins 6, 16 and 17 are normally expressed in epithelial appendages, such as the hair follicle (McGowan and Coulombe, 1998). In this setting, loss of keratin 17 was revealed to

result in an early entry into the catagen (involution) phase of the hair cycle, which is mediated by physiological apoptosis (McGowan et al., 2002). *In vitro* and *in vivo* data demonstrated that the loss of keratin 17 lead to an increase in TNFα signaling, suggesting that K17 participates in the regulation of the hair cycle via regulation of apoptosis (McGowan et al., 2002; Tong and Coulombe, 2006).

Keratins as Modulators of Cell Growth

Another cellular function in which keratins are involved is cell growth. One way in which keratins influence cell growth is by modulating protein synthesis by interacting with protein elements of the translational machinery (Hesketh and Pryme, 1991). An example of this is the direct interaction of keratin 17 with the adaptor protein 14-3-3σ (Kim et al., 2006). Protein synthesis is abnormally low in keratin 17 null keratinocytes stimulated to grow, correlating with reduced activity of the Akt-mTOR signaling axis (Kim et al., 2006). Phosphorylation of keratin 17 on serine 44 by the growth-promoting kinase p90 RSK1 may represent the key molecular event regulating the interaction involving keratin 17 and 14-3-3σ, and the associated impact on cell growth (Pan et al., 2011). Additionally, keratin 17 (along with other skin-expressed keratins) was shown to interact directly with eukaryotic elongation factor 1γ, illustrating another mechanism through which keratins modulate protein synthesis (Kim et al., 2007).

There is also evidence that keratins 8 and 18 regulate protein synthesis in the liver (Galarneau et al., 2007). Hepatocytes from livers of keratin 8-null mice exhibit a reduced rate of protein synthesis thought to be due to perturbed plectin/RACK1 distribution

(Galarneau et al., 2007).   Further information on the regulations of protein synthesis by keratins was revealed through the ablation of the entire type II keratin gene cluster on chromosome 15 in mice, effectively generating a complete keratin null mouse model (Vijayaraj et al., 2009). In this model, it was discovered that keratins modulate protein synthesis and regulate the cellular localization of glucose transporters GLUT-1 and -3 (Vijayaraj et al., 2009). In this setting, keratins were also revealed to play a role in vasculogenesis and hematopoiesis in the developing embryo, providing further evidence for keratins modulating cell growth (Kellner and Coulombe, 2009; Kim and Coulombe, 2010; Kroger et al., 2013).

Role of Keratins in Cell Migration

Another cellular function in which keratins are involved is motility. Perhaps the best example of keratins' involvement in motility is provided by tissue repair following injury to the skin. In this context, keratinocytes originating from the edges of a wound must migrate to participate in its re-epithelialization and restoration of the vital barrier properties. Within hours after injury to the epidermis, expression of keratins 6, 16, and 17 is induced at the expense of the normal differentiation-related keratins 1 and 10 (Paladini et al., 1996). Expression of these wound-inducible keratins correlates with a massive reorganization of the keratin filament network, which impacts a number of cellular processes related to re-epithelialization, including cell growth as related above (Paladini et al., 1996; Kim et al., 2006). Seemingly paradoxically, genetic ablation of keratin 6 results in an increased migration phenotype in various *ex vivo* culture paradigms (Wong and Coulombe, 2003). This enhanced migration is due to the activation of Src, a

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kinase that is physically bound to and negatively regulated by keratin 6 (Rotty and Coulombe, 2012). In the case of keratins 8 and 18, keratins and their associated proteins modulate migration by binding to upstream effectors of cell migration, such as protein kinase C (PKC) and focal adhesion kinase (FAK) (Bordeleau et al., 2010).

Keratins Modulate Skin Pigmentation

Several clinical findings have revealed a role for keratins in the pigmentation process of the skin. EBS with mottled pigmentation (EBS-MP) is a rare subtype of EBS disease often caused by a specific mutation in the *KRT5* gene (P25L) (Uttam et al., 1996). EBS-MP presents with 2-5mm hyper- or hypo-pigmented spots in addition to blistering (Fischer and Gedde-Dahl, 1979). Additionaly, EBS Migrating (EBS-Migr) is another rare EBS subtype presenting with lesions which heal with increased pigmentation (Gu et al., 2003). Several non-EBS skin disorders also present with pigmentation defects. One such disorder is Dowling-Degos disease (DDD), which occurs as a result of K5 haploinsufficiency (Dowling and Freudenthal, 1938). Also, Naegeli–Franceschetti–Jadassohn syndrome and dermatopathia pigmentosa reticularis are disorders caused by mutations in keratin 14 which also present with aberrant skin pigmentation patterns. Finally, a mutagenesis screen in mice revealed a role for keratins 1 and 2e in pigmentation, with mutants developing hyperkeratosis and epidermal thickening followed by hyperpigmentation (Fitch et al., 2003). The mechanism of keratins modulating the complex process of skin pigmentation is still unclear, and remains an active area of research (Gu and Coulombe, 2007).

Keratins and Cancer

Several early studies in cancer biology hinted at roles for keratins in this setting. For example, analysis of cancerous lesions from a number of sources revealed that keratin immunoreactivity was detectable in epithelial neoplasms, but not in non-epithelial cancers such as mammary adenocarcinomas (Schlegel et al., 1980; Gabbiani et al., 1981; Ramaekers et al., 1981). Later, it was determined that keratins 5, 6, and 17 were consistently induced in cancers of basal cell origin (Ordonez, 1998; Markey et al., 1992). In fact, keratin 17 was shown to correlate with poorer patient prognosis in breast cancer (van de Rijn et al., 2002) and epithelial ovarian cancer (Wang et al., 2013). Given the consistent expression of keratins in cancers as well as the roles keratins are now known to fulfill in signaling pathways, it is plausible that keratins are more than mere cellular bystanders in this type of condition. The overexpression of keratin 8 in the skin epithelia of transgenic mice provided evidence in support of a positively- influential role for keratins in tumorigenesis (Casanova et al., 2004). These animals presented with an apparent disruption of the differentiation of the skin, as well as severe dysplasia of the hair follicles and an increase in the progression of papillomas toward malignancies (Casanova et al., 2004). Another cancer context which consistently correlates with the expression of keratin 17 is basal cell carcinoma (BCC), which is caused by aberrant sonic hedgehog (Shh) signaling and is the most common cancer in European descendants (Epstein, 2008). Development of BCC-like lesions in mouse models with constitutive expression of a downstream Shh effector, Gli2, was examined in the presence and absence of a functional *Krt17* locus (Depianto et al., 2010). In this setting, tumor onset

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Table 1.1: Proposed Functions for Keratin 5

Cellular Function	Comments	Reference(s)
Structural Support	Proper structure and organization of intracellular keratin filaments is essential to withstanding frictional and mechanical stresses.	Vassar et al., 1991; Coulombe et al., 1991b; Lloyd et al., 1995; Peters et al., 2001
Skin Pigmentation	Diseases EBS-MP, EBS-Migr, and DDD are caused by mutations at the *KRT5* locus in humans, and present with aberrant pigmentation.	Fischer et al., 1979; Gu et al., 2003; Dowling and Freudenthal, 1938; Betz et al., 2006; Uttam et al., 1996
Langerhans Cell Density	Ablation or mutation of *KRT5* results in an increased Langerhans cell density in the epidermis of mice and EBS patients.	Roth et al., 2009
Increased BCC Susceptibility	Recent genome-wide association study identified two missense mutations in *KRT5* that correlate with increased BCC susceptibility.	Stacey et al., 2009

List of functions ascribed to keratin 5. EBS, Epidermolysis Bullosa simplex; EBS-MP, EBS with mottled pigmentation; EBS-Migr, EBS Migratory; DDD, Dowling-Degos disease; BCC, basal cell carcinoma.

was significantly delayed in the absence of keratin 17. This delay correlated with decreased cell proliferation and reduced inflammation (Depianto et al., 2010). To explore this further, specific markers of inflammation were assessed, and it was concluded that the loss of keratin 17 caused a polarization of the immune response from what should be a Th1/Th17-dominated profile to a Th2-dominated immune profile in $GLI2^{Tg}$, $Krt17^{-/-}$ skin (Depianto et al., 2010; Pan et al., 2013). These findings suggest immune modulation as a potential mechanism behind the K17-dependent modulation of tumor formation in mouse skin. Interestingly, the loss of keratin 5 also results in an alteration of immune status in neonatal mouse skin, resulting in an increase in specific cytokines such as Ccl2, Ccl19, and Ccl20, which are involved in the recruitment of Langerhans cells (Roth et al., 2009). Additionally, a recent genome-wide association study identified two variants of keratin 5 as markers of increased susceptibility to basal cell carcinoma, an intriguing finding that is examined in Chapter 3 (Stacey et al., 2009).

Goal of this Dissertation

It is clear from this wealth of data that keratins fulfil both structural support functions and non-mechanical functions in a number of epithelial contexts. The intent of this dissertation is to present data that can hopefully shed further light on these biological functions, and perhaps reveal novel roles for keratins in epithelial cell biology. Chapter 2 of this dissertation will address ongoing efforts to explore the contribution of intrinsic determinants of keratin filament bundling to their structural support *in vivo*, and Chapter 3 will present ongoing efforts to explore the properties of the newly defined variants of keratin 5 that are associated with a modestly increased risk of developing BCC.

CHAPTER 2

DIRECTED EXPRESSION OF A CHIMERIC TYPE II KERATIN PARTIALLY

RESCUES KERATIN 5 NULL MICE

David M. Alvarado and Pierre A. Coulombe

ABSTRACT

The crucial role of structural support fulfilled by keratin intermediate filaments (IFs) in surface epithelia likely requires that they be organized into cross-linked networks. For IFs comprised of keratins 5 and 14, which occur in basal keratinocytes of the epidermis, formation of cross-linked bundles is, in part, self-driven through cis-acting determinants. Here, we targeted the expression of a bundling-competent *KRT5/KRT8* chimeric cDNA (*KRT8bc*), or bundling-deficient wildtype *KRT8* as a control, to the epidermal basal layer of *Krt*5 null mice to assess the functional importance of keratin IF self-organization *in vivo*. We report that targeted expression of K8bc rescued *Krt5* null mice with a 47% frequency, while K8 completely failed to do so. This outcome correlated with lower than expected levels of K8bc and especially K8 mRNA and protein in the epidermis of E18.5 replacement embryos. *Ex vivo* culture of embryonic skin keratinocytes confirmed the ability of K8bc to form IFs in the absence of K5. Additionally, electron microscopy analysis of E18.5 embryonic skin revealed that the striking defects observed in keratin IF bundling, cytoarchitecture, and mitochondria are partially restored by K8bc expression. As young adults, viable *KRT8bc* replacement mice develop alopecia and chronic skin lesions, indicating that the skin epithelia are not completely normal. These findings are consistent with a contribution of self-mediated organization of keratin IFs to structural support and cytoarchitecture in basal layer keratinocytes of the epidermis, and underscore the importance of context-dependent regulation for keratin genes and proteins *in vivo*.

INTRODUCTION

The contribution of intermediate filaments (IFs) to the maintenance of cell and tissue integrity is essential in surface epithelia (e.g. skin, oral mucosa), muscle, and other tissues routinely subjected to stress. Loss or disruption of this function accounts for the cell and tissue fragility that underlies a large number of genetically-determined diseases that are individually rare but debilitating (Omary et al., 2004; Szeverenyi et al., 2008; Coulombe et al., 2009). Epidermolysis bullosa simplex (EBS), for instance, is a condition in which the basal cell layer of epidermis and related stratified epithelia ruptures in response to trivial frictional trauma, and is associated with dominantly-acting mutations in either keratins 5 or 14 (K5 or K14), the main keratin pairing that is expressed in this epithelial setting (Bonifas et al., 1991; Coulombe et al., 1991a; Lane et al., 1992). EBS is the first among more than 80 IF-based disorders (Szeverenyi et al., 2008). There is as yet no effective treatment for EBS and other disorders rooted in defective IFs (McLean and Moore, 2011; Coulombe and Lee, 2012).

The role of keratin IFs towards the maintenance of cell and tissue integrity is a composite function of their unique micromechanical properties (Janmey et al., 1991; Ma et al., 2001) and extensive integration within the cytoskeleton and cell adhesion apparati (Fuchs and Cleveland, 1998; Coulombe and Wong, 2004; Kroger et al., 2013). In particular, this function reflects several key attributes of keratin IFs, including their intracellular abundance (Feng et al., 2013), their organization as cross-linked networks (Ma et al., 2001; Yamada et al., 2002), and their attachment at sites of cell-cell and cell-matrix adhesions (Fuchs and Cleveland, 1998; Coulombe and Wong, 2004) and at the

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surface of the nucleus (Wilhelmsen et al., 2005). The molecular basis for IF anchorage at sites of integrin-based cell-matrix adhesions and cadherin-based cell-cell adhesions is quite well understood (Simpson et al., 2011; Suozzi et al., 2012). By comparison, the basis for the cross-linked organization of IFs is poorly understood in most types of cells. In epidermal and hair keratinocytes, for instance, the filament bundling-promoting influence of filaggrin (Steinert and Marekov, 1995) and trichohyalin (Lee et al., 1993) is well-established but restricted to late stages of terminal differentiation, whereas the influence of plectin, epiplakin, and related plakin family members (Stappenbeck et al., 1993; Jang et al., 2005; Green et al., 2005) and/or inter-filament disulfide bonding (Lee et al., 2012; Sun and Green, 1978) remains unclear.

Keratin IFs assembled from the type II K5 (590 residues; ~58 kDa in humans (Lersch and Fuchs, 1988)) and type I K14 (472 residues; ~50 kDa in humans (Hanukoglu and Fuchs, 1983)) exhibit the remarkable property of self-organization into cross-linked networks, both as purified entities *in vitro* (Wilson et al., 1992; Bousquet et al., 2001; Ma et al., 2001; Lee and Coulombe, 2009) and when expressed in keratin-free fibroblasts (Lee and Coulombe, 2009). The property of self-organization enhances the mechanical resilience of K5-K14 filament assemblies, and depends on interactions involving the distal 25 amino acids of K14's C-terminal domain, which is exposed at the filament surface, and two separate regions within K5, the so-called head-1A (70 residues) and L2-2B subdomains (129 residues)(Lee and Coulombe, 2009; Kim and Coulombe, 2010). The property of self-organization appears to be specific to natural keratin pairings (e.g., K5-K14 and K8-K18 (Yamada et al., 2002)), but is transferable upon "chimeragenesis".

For instance, K8 (511 residues; ~54 kDa in humans (Leube et al., 1986)), a type II keratin normally restricted to simple epithelial linings, readily co-polymerizes to yield 10nm filaments when paired with K14, but the resulting assemblies cannot undergo self-organization, either *in vitro* or in transfected cells (Lee and Coulombe, 2009). Transferring the head-1A and L2-2B subdomains from K5 into K8 confers the property of self-organization to the resulting chimera (designated K8bc) when artificially paired with K14, *in vitro* as well as in transfected fibroblasts (Lee and Coulombe, 2009).

Lee and Coulombe (Lee and Coulombe, 2009) predicted that the property of self-organization is a significant determinant of K5-K14's ability to provide structural support in the basal layer of the epidermis. A corollary from this prediction is that targeted expression of the K8bc chimera, but not wildtype K8, to basal layer keratinocytes should rescue the extensive skin blistering and perinatal lethality phenotype of mice carrying a null mutation at the *Krt5* locus (Peters et al., 2001). Here, we report on our effort to carry out such an experiment using *Krt5* null mice.

EXPERIMENTAL PROCEDURES

Cells and transgenic mouse lines. All protocols involving mice were approved by the Johns Hopkins University Animal Care and Use Committee (Baltimore, MD). Mouse lines were maintained under specific pathogen-free (SPF) conditions, and fed chow and water *ad libitum*. The plasmids pIRES2-GFP hK8bc and pIRES2-GFP hK8 (Lee and Coulombe, 2009) were digested with EcoRI and NheI (New England Biolabs), and the resulting cDNA inserts were treated with calf intestinal phosphatase (New England

Biolabs), gel-purified using QIAquick gel extraction kit (Qiagen), and subcloned into the

BamHI site of the modified *KRT14* expression vector (Saitou et al., 1995) containing

approximately 2500 base pairs (bp) of 5' upstream and approximately 700 bp of 3'

downstream *KRT14* sequence, as well as a rabbit *β-globin* intron for mRNA stability.

Clones of the correct orientation were transiently transfected into cell line NIH-3T3

(ATCC CRL-1658) with GeneJuice® transfection reagent (EMD Millipore) for

preliminary observations. Plasmids were then linearized with KpnI and AseI (New

England Biolabs), and the final linearized ~5kb DNA fragment was used to generate

transgenic mice by pronuclear injection. Genomic DNA was isolated from potential

founders at approx. 6 weeks of age, and the DNA was subjected to PCR-based

genotyping with Accustart™ II mouse genotyping kit (Quanta Biosciences) according to

manufacturer's instructions, using the primers 5'- TGCATATAAATTCTGGCTGGCG-

3' (forward) and 5'- GCATGAACATGGTTAGCAGAGGG-3' (reverse), which are

directed to the rabbit β-globin intron contained within the expression vector. All

subsequent progeny were genotyped using this method.

Krt5 null mice were previously described (Peters et al., 2001; Kerns et al., 2007),

and were maintained in a mixed genetic background. These mice were genotyped using a

multiplex PCR assay using the universal forward primer 5'-

CCCACTAATCATTCACAGCTCG-3', *Krt5* reverse primer 5'-

ACCAAAACCAAATCCACTGCCG-3', and *HPRT* reverse primer 5'-

CGAGTCTGAAGCTCTCGATTTCC-3'. Replacement mice were generated by

breeding *KRT8bc$^{Tg/-}$* or *KRT8$^{Tg/-}$* mice with *Krt5$^{+/-}$* mice to yield *KRT8bc$^{Tg/-}$Krt5$^{+/-}$* or

KRT8[Tg/-]*Krt5*^{+/-} mice, respectively. These mice were then bred with *Krt5*^{+/-} mice to yield

the following genotypes: *Krt5*^{+/+} or Krt5^{+/-} (control), *KRT8bc*[Tg/]*Krt5*^{+/+} or *KRT8*[Tg/-]

Krt5^{+/+} (referred to herein as *KRT8bc* or *KRT8* transgenic mice), *Krt5*^{-/-} (*Krt5* null), and

KRT8bc[Tg/-]*Krt5*^{-/-} or *KRT8*[Tg/-]*Krt5*^{-/-} (referred to herein as *KRT8bc* or *KRT8* replacement

mice).

Statistical analyses. All statistics were performed using Graphpad Prism

software. One- or two-way analyses of variance (ANOVA) were used to test for

significance where appropriate, and adjusted P values were reported.

Protein purification and analysis of transgene expression. Plasmids pET-K5 and

pET-K8 (Lee and Coulombe, 2009) were transformed into the *E. coli* strain BL21 (DE3)

to produce the corresponding recombinant proteins as inclusion bodies (Coulombe and

Fuchs, 1990; Lee and Coulombe, 2009). These recombinant proteins were purified as

previously described (Ma et al., 2001; Yamada et al., 2002) using Hi-TrapQ and MonoQ

ion exchange chromatography columns (GE Healthcare). Protein concentrations were

determined using a Bradford assay kit (Bio-Rad), and serial dilutions were performed to

yield standards of known concentration for analyses outlined below.

For analysis of transgene expression in development stage 18.5 (E18.5) embryos,

pregnant mice were sacrificed 18 days post-timed mating and the skins of the embryos

were harvested. Skins were chopped repeatedly with a razor blade and urea-soluble

proteins were then extracted as previously described (Paladini and Coulombe, 1998).

Protein concentrations were determined with a Bradford assay kit (Bio-Rad). To

determine expression levels of endogenous K5 protein and of transgenic proteins, standards of known concentration were run alongside E18.5 samples. Otherwise, equal quantities of protein from genotypes of interest were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and antibody incubations were performed in 5% milk in 1x TBS-T. Protein epitopes were detected with the following antibodies: rabbit anti-K5 AF-138, (Covance) rabbit anti-K14 AF-64 (Covance), chicken anti-human K8 antibody ab107115 (Abcam), and mouse anti-GAPDH antibody sc-365062 (Santa Cruz Biosciences). Secondary antibodies used were horseradish peroxidase- conjugated goat anti-rabbit, goat anti-chicken, and goat anti-mouse. Blots were developed using the SuperSignal West Pico Chemiluminescent substrate kit and were imaged with FluorChem® Q MultiImage III (Alpha Innotech). Band signal intensity was measured from these images with the AlphaView® Q software. Standard curve line of best fit equation and R^2 values were calculated using Graphpad Prism. Calculation of K5, K8bc and K8 quantities was performed using the standard curve equations in Microsoft Excel.

For analysis of transgenic protein expression in adult animals, wildtype and transgenic animals were sacrificed at 6wks of age and the back skins were harvested. The tissue was snap-frozen and urea-soluble proteins were extracted as previously described (Paladini and Coulombe, 1998). Equal quantities of urea-soluble protein were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were developed using the SuperSignal West Pico Chemiluminescent substrate kit and were

imaged with FluorChem® Q MultiImage III (Alpha Innotech). All images were inverted and cropped using ImageJ software.

Quantitative real-time PCR. Total RNA from either 6wk adult animals or E18.5 embryos was isolated using TRIzol® reagent according to manufacturer's instructions. Complementary DNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR® Green Real-Time PCR Master Mix from Life Technologies on a Bio-Rad C1000™ thermocycler and CFX™ Real-Time System. Primers used were directed toward *KRT8* (5'-CAAGGTGTCCACCTCTGGC-3' and 5'-ATAGCCGCCGCCCAGGCCA-3'), *Krt14* (5'-AGCGGCAAGAGTGAGATTTCT-3' and 5'-CCTCCAGGTTATTCTCCAGGG-3') *Actb* (5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3') and *Gapdh* (5'-AAATGGTGAAGGTCGGTGT-3' and 5'-ACTCCACGACATACTCAGCAC-3'). Delta Cq (ΔCq) and delta-delta Cq ($\Delta\Delta$Cq) for each target was determined by first subtracting the averaged Cq values for Actin and GAPDH from the Cq of the target, and then by subtracting the ΔCq of the wildtype sample from each of the other samples when applicable. Relative RNA quantity was calculated by taking $2^{-\Delta Cq}$, and relative fold change was calculated by taking $2^{-\Delta\Delta Cq}$. Calculations were performed using Microsoft Excel.

Histopathology. Phenotypic adult *KRT8bc* replacement and control mice were harvested at various ages for morphological study. Prior to sacrifice, images of the mice were taken with a Samsung Digimax S500 digital camera. Tissues analyzed included

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hairy skin, hairless skin (if present), ulcerative skin, tongue, forepaws, and tail. Tissues

were fixed overnight in Bouin's fixative (Bio-Rad), dehydrated, and processed for

paraffin embedding by AML Laboratories. Sections were cut 5μm thick and stained with

hematoxylin and eosin. Images were taken on a Zeiss AxioObserver inverted microscope

with an AxioCam HRc camera. Images were cropped and scale bars were added in

ImageJ.

Immunofluorescence. For analysis of cultured keratinocytes, E18.5 embryos were

again harvested and the skins removed. The keratinocytes were isolated using a modified

version of the protocol reported by Reichelt and Haase (Reichelt and Haase, 2010). After

incubation with dispase solution, keratinocytes were isolated as previously reported

(Bernot et al., 2005). Keratinocytes were cultured on collagen I-coated coverslips in

CnT-57 media (CellnTec) supplemented with Pen/Strep at 37°C and 5% CO_2 in the

absence 3T3-J2 feeder cells. Sub-confluent coverslips were fixed with ice-cold methanol

at -20°C or with 3.3% paraformaldehyde (PFA) at room temperature for 10 min.

Coverslips fixed with PFA were extracted with 1% Triton X-100 in PBS buffer for 5 min

at room temperature. Antibodies used to detect K5 and K14 were described above. K8

was detected with chicken anti-human K8 antibody ab14053 (Abcam). Secondary

antibodies used were goat anti-chicken Alexa-488 and goat anti-rabbit Alexa-594, and

nuclei were stained with Hoechst fluorescent dye. Images were taken on a Zeiss

AxioObserver inverted microscope with an AxioCam MRm camera.

For analysis of adult transgenic back skin, animals were harvested as described

above and a portion of the back skin was embedded in Tissue-Tek® O.C.T. media

(Sakura) and frozen in liquid nitrogen. 5μm thick frozen sections were cut and washed

once with PBS buffer before probing with the antibodies described above. Images were

taken on a Zeiss AxioObserver inverted microscope with an AxioCam MRm camera. All

images were cropped and scale bars were added using ImageJ.

Electron microscopy of skin tissue. Skins from 6wk adult animals or E18.5

embryos were harvested and immersed in 2% paraformaldehyde/2% glutaraldehyde for

48hrs. After primary fixation, tissue samples were post fixed in 1% osmium tetroxide in

0.1M cacodylate buffer for 1 hour on ice. Tissue samples were then rinsed and en bloc

stained with 2% aqueous Uranyl acetate for 1 hour at room temperature. Samples were

then dehydrated in a graded series of ethanol (50%, 70%, 95% and 100%). Tissue was

cleared in propylene oxide and then infiltrated in a graded series of Epon 812/ propylene

oxide (50/50 and 70/30) for one hour and overnight, respectively, followed by two

changes of pure Epon 812, and then polymerized for 48 hours at 60ºC. Semithin sections

(1μm) were cut using glass knives on a Sorvall MT2B Ultramicrotome. Sections were

mounted on glass slides and stained with 1% Toluidine blue in 1% Sodium borate and

coverslipped with Permount. Areas of interest were selected and thin sections (60nm-

80nm) were cut using a diamond knife. The sections were mounted on formvar-carbon

coated 150 mesh copper grids and were post-stained with 2% aqueous Uranyl acetate and

Reynold's Lead citrate (REYNOLDS, 1963). Sections were analyzed using a Hitachi

HU12A electron microscope. Kodak Electron Microscope Film 4489 was developed and

negatives were scanned using an Epson Perfection V500 Photo Scanner. Images were

cropped and scale bars were added in ImageJ. For cell and nuclear aspect ratio analysis,

lines were drawn from the basement membrane to the top of the cells (height) and from side to side (width) at the greatest distance for each cell measured using ImageJ. The lines were measured and the height was divided by the width. Basal cell desmosomes, hemidesmosomes, and mitochondria were assessed individually from 5000x images.
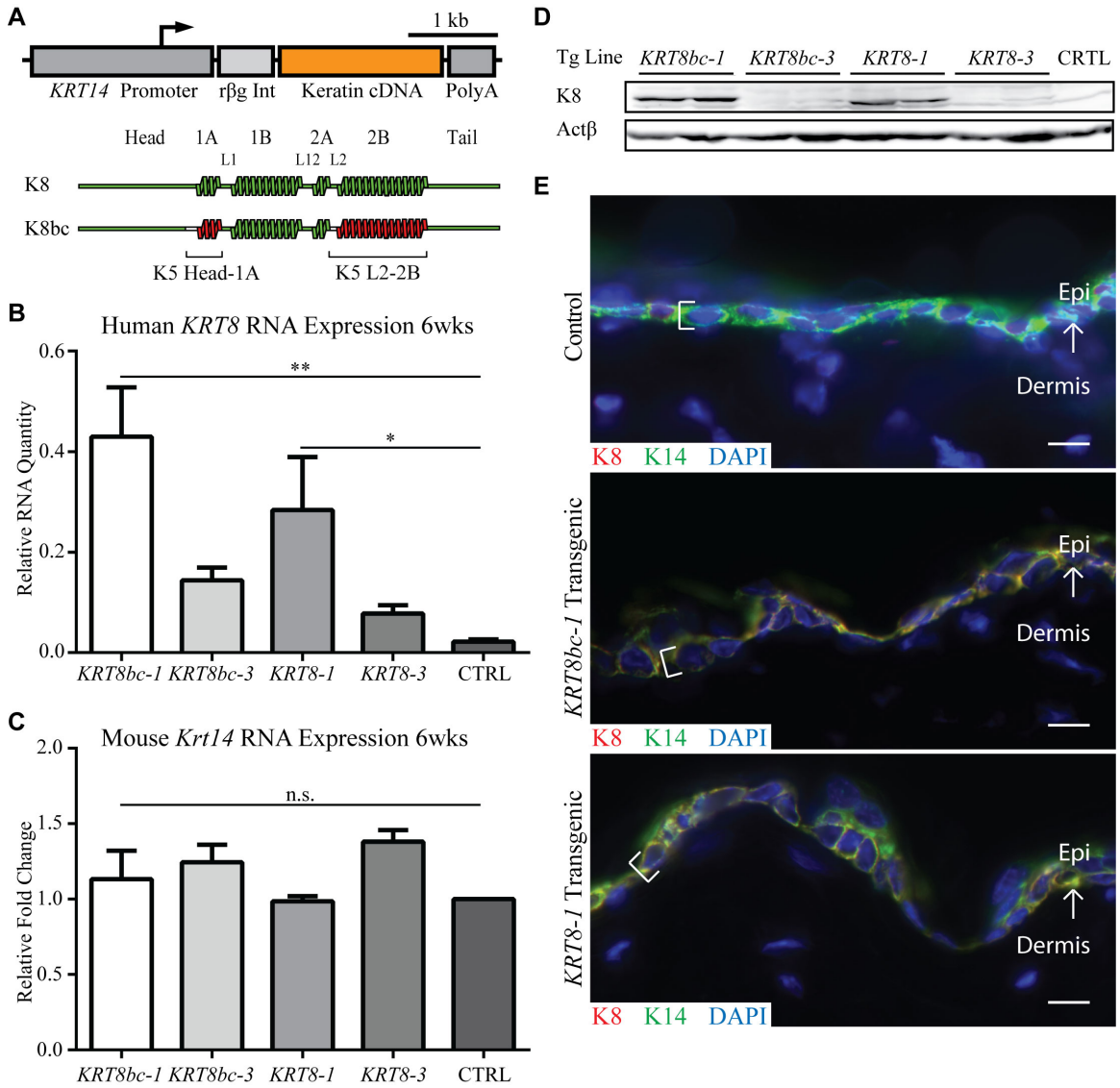
RESULTS

KRT8 *and* KRT8bc *transgenes are expressed in transgenic mouse epidermis.* The cDNAs encoding wildtype human K8 (Yamada et al., 2002) and the K8bc chimera (Lee and Coulombe, 2009) were subcloned into the *KRT14* gene promoter-based expression vector (Saitou et al., 1995) as described (Paladini and Coulombe, 1998). Transfection of the final DNA constructs (Fig. 2.1A) into NIH-3T3 cells, along with wildtype *KRT14*, gave rise to keratin IFs (data not shown), indicating that K8 and K8bc can each co-polymerize with K14 as reported (Lee and Coulombe, 2009). The two constructs were isolated and used to generate transgenic founders by pronuclear injection. Transgene-positive founders were then bred to establish transgenic mouse lines. For each construct, the two lines showing the highest transgene copy number in a single insertion site (based on internally-controlled PCR and frequency of F1 transgenic offspring; data not shown) were retained for further analysis.

The resulting progeny from these transgenic lines, designated *KRT8bc-1*, *KRT8bc-3*, *KRT8-1*, and *KRT8-3* respectively, did not exhibit any visible phenotype. Back skin tissue was harvested from age- and sex-matched animals from these lines for analysis of transgene expression. Of these four lines, two expressed transgene mRNA at

Figure 2.1: Generation and Characterization of Transgenes. A) Schematic depiction of the transgenes used in this study. The human *KRT14* gene promoter was used to drive the tissue-specific expression of keratin cDNAs (*KRT8*, *KRT8bc*), while the rabbit beta-globin intron and human *KRT14* 3'UTR sequence ("PolyA") serve to stabilize the transgene mRNA in mouse cells. B, C) qRT-PCR analysis by of transgenic human *KRT8* mRNA (B), and endogenous mouse *Krt14* mRNA (C), in back skin harvested from age- and sex-matched 6-weeks old animals. Relative RNA amount is normalized to both actin and GAPDH. CRTL indicates $Krt5^{+/+}$ skin. Error bars represent standard error of the mean. A one-way ANOVA (Dunnett's test) was used to test for significance, and the adjusted P values are reported. n.s., not significant; *, $P<0.04$; **, $P<0.002$. D) Analysis of total skin protein extracts (10µg/lane) by Western blot in 6wk old sex-matched adult animals. Two mice are analyzed for each of four transgenic lines; CRTL indicates $Krt5^{+/+}$ skin. E) Analysis of transgene expression in frozen skin sections of 6wk old sex-matched adult animals. K8 epitopes are only present in basal layer keratinocytes (see brackets) of *KRT8bc-1* and *KRT8-1* transgenic epidermis, and co-localizes with endogenous K14 in a normal keratin filament network. Control indicates $Krt5^{+/+}$ skin. Arrows depict the interface between the epidermis (Epi) and dermis. Bars = 10µm.

Figure 2.1: Generation and Characterization of Transgenes.

appreciable levels by qRT-PCR: lines *KRT8bc-1* and *KRT8-1* (Fig. 2.1B). Expression of

Krt14 mRNA was unaffected relative to wildtype (Fig. 2.1C). Western blot analyses

assessing protein expression revealed similar results, with appreciable and comparable

levels of transgenic protein detectable for lines *KRT8bc-1* and *KRT8-1* only (Fig. 2.1D).

Analysis of frozen sections of mouse back skin by immunofluorescence confirmed that

K8-specific epitopes occurred in the basal layer of the epidermis, as expected (Fig. 2.1E).

The *KRT8bc-1* and *KRT8-1* lines were selected to test whether *KRT14* promoter-driven

expression of K8 or K8bc protein can rescue the *Krt5* null phenotype.

The KRT8bc*, but not the* KRT8 *transgene, is able to rescue the* Krt5 *null*

phenotype. Krt5 null mice exhibit severe fragility and blistering of the skin, and die with

complete penetrance within the first hour of birth (Peters et al., 2001), providing a clear

readout for the ability of other type II keratins (e.g., K8, K8bc) to functionally substitute

for endogenous K5. The *KRT8bc-1* and *KRT8-1* transgenic mice (Fig. 2.1) were each

bred to *Krt5* hemizygous null (*Krt5*$^{+/-}$) mice. Once sexually mature, the resulting

KRT8bc$^{tg/-}$*Krt5*$^{+/-}$ and *KRT8*$^{tg/-}$*Krt5*$^{+/-}$ offspring were crossed with *Krt5*$^{+/-}$ mice. While

this strategy ensures a uniform number of rescue transgene copies within the progeny,

only 1 of every 8 offspring is predicted to be a candidate for phenotypic rescue (*KRT8bc*

or *KRT8* replacement mice). Replacement events wherein the mouse survived to

weaning age (P21) were scored as successful rescues. Of 411 live births analyzed, 16 out

of 34 mice with the *KRT8bc* replacement genotype (47%) survived to weaning (Table

2.1). The surviving *KRT8bc* replacement animals did not show any detectable blistering

at the time of birth, and grew normally to adulthood, indicating the transgene could

Table 2.1: Summary of Type II Keratin Protein Replacement Experiment in Mouse.

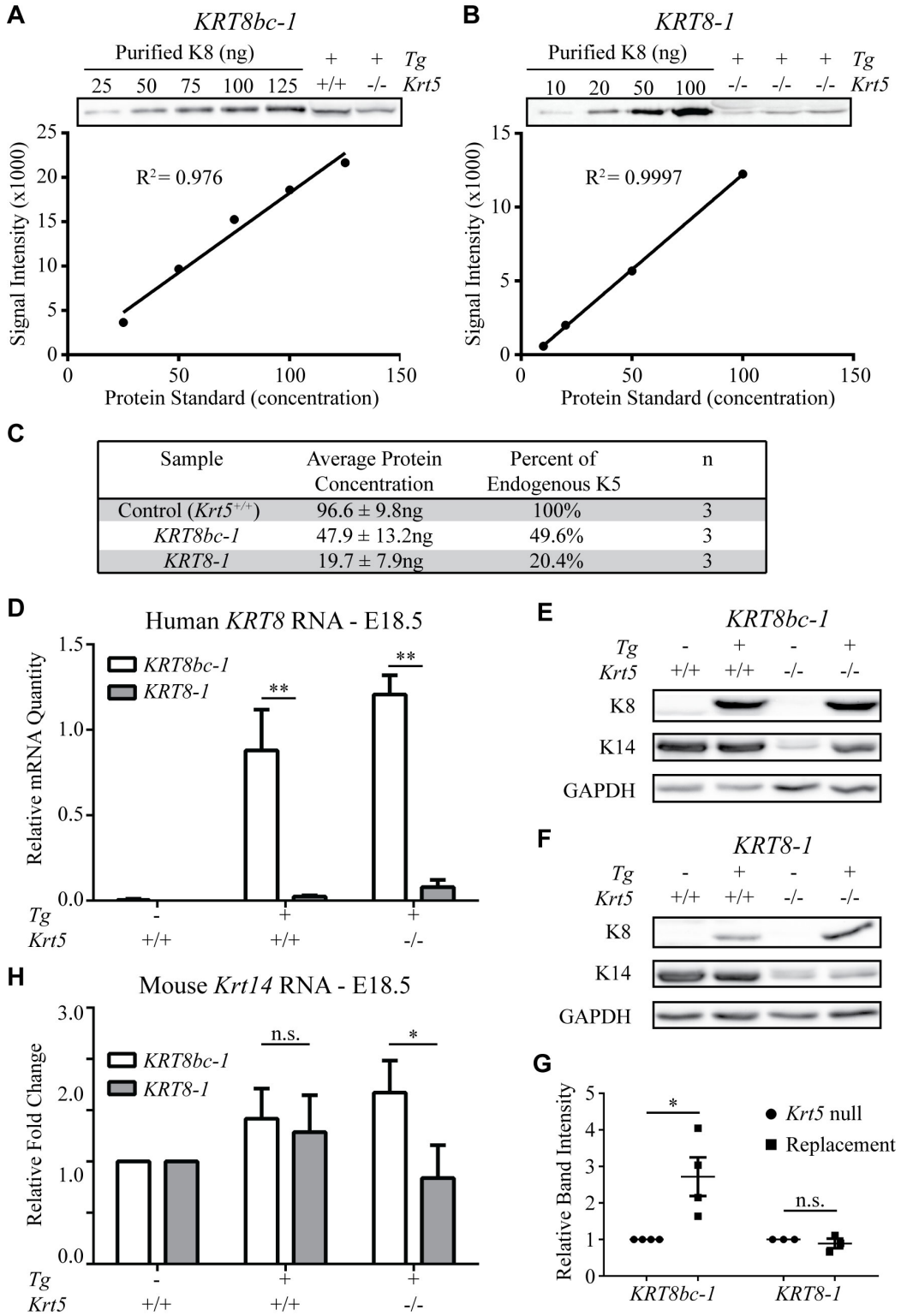| Transgenic Line	No. Live Births	No. Animals Genotyped	No. Replacement Animals	No. Viable Replacements	Percent Viable Replacements
KRT8bc-1	411	361	34	16	47%
KRT8-1	191	180	11	0	0%

Progeny from the replacement breeding strategy (see Methods) were monitored at and following birth. "Viable replacement" implies that the mouse survived at least until weaning.

functionally substitute for endogenous K5. By contrast, none of the 11 mice born with

the *KRT8* replacement genotype from 191 live births showed rescue (Table 2.1); in fact,

none lived beyond the first few hours after birth, demonstrating that the *KRT8* transgene

cannot rescue the K5 deficiency.

Expression of K8bc and K8 replacement proteins and mRNAs in the skin of E18.5

Krt5 *null mice.* Next, we assessed the amount of K8bc and K8 replacement proteins in

the skin of *KRT8bc* and *KRT8* replacement mice, relating them to the levels of K5 protein

in control mice. This analysis was conducted in E18.5 embryos, just prior to birth, to

enable a direct comparison between the two types of rescue proteins and to avoid the

complications stemming from the extensive skin blistering seen in *Krt5* null and *KRT8*

replacement mice. Purified recombinant proteins were used to generate standard curves

for K5 and K8 epitopes (see Methods), enabling the determination of transgenic protein

amount in these lines (Fig. 2.2A, B and data not shown). Using this strategy we found

that endogenous K5 is expressed at 96.6ng per 10μg of total skin proteins in control

E18.5 embryos (n=3; Fig. 2.2C). *KRT8bc* replacement embryos express 47.9ng K8bc

protein per 10μg of total protein extract (n=3), corresponding to ~50% of the

concentration of endogenous K5 in control embryos (Fig. 2.2C). The *KRT8* replacement

embryos were determined to express 19.7ng K8 protein per 10μg of total protein extract

(n=3), accounting for ~20% of endogenous K5 levels (Fig. 2.2C). The difference

between K8bc and K8 expression is also observed at the mRNA level in E18.5 mouse

embryos (Fig. 2.2D). We infer that the "half-normal" levels of K8bc protein may

explain, in part, the partial rescue (47% frequency) observed in *KRT8bc* replacement

Figure 2.2: Analysis of Transgene Expression in E18.5 Mouse Embryos. A, B) Quantification of transgenic protein expressed in lines *KRT8bc-1* (A) and *KRT8-1* (B) using standard curves of purified recombinant human K8. Line of best fit and R^2 values were calculated using Graphpad Prism. C) Summary of the transgenic protein quantification from multiple biological (n) and technical replicates. D) Transgene RNA expression levels for lines *KRT8bc-1* and *KRT8-1* in E18.5 skin. E, F) Expression of transgenic and endogenous proteins for lines *KRT8bc-1* (E) and *KRT8-1* (F) in E18.5 skin is shown by western blot analysis. G) Intensity of K14 protein signal relative to loading control signal was quantified, and the increase of K14 in the replacement mouse skin setting is illustrated. H) Endogenous *Krt14* RNA expression levels for lines *KRT8bc-1* and *KRT8-1* in E18.5 skin. RNA levels are illustrated relative to Actin and GAPDH. Error bars represent standard error of the mean. A two-way ANOVA (Sidak's test) was used to test for significance, and the adjusted P values are reported. n.s., not significant; *, $P<0.005$; **, $P<0.0001$.

Figure 2.2: Analysis of Transgene Expression in E18.5 Mouse Embryos.

A

KRT8bc-1

Purified K8 (ng)

| 25 | 50 | 75 | 100 | 125 | + +/+ | + -/- | *Tg Krt5* |

$R^2 = 0.976$

Signal Intensity (x1000)

Protein Standard (concentration)

B

KRT8-1

Purified K8 (ng)

| 10 | 20 | 50 | 100 | -/- | -/- | -/- | *Tg Krt5* |

$R^2 = 0.9997$

Signal Intensity (x1000)

Protein Standard (concentration)

C

Sample	Average Protein Concentration	Percent of Endogenous K5	n
Control ($Krt5^{+/+}$)	96.6 ± 9.8ng	100%	3
KRT8bc-1	47.9 ± 13.2ng	49.6%	3
KRT8-1	19.7 ± 7.9ng	20.4%	3

D

Human *KRT8* RNA - E18.5

Relative mRNA Quantity

KRT8bc-1
KRT8-1

**   **

Tg   -   +   +
Krt5   +/+   +/+   -/-

E

KRT8bc-1

| *Tg* | - | + | - | + |
| *Krt5* | +/+ | +/+ | -/- | -/- |

K8

K14

GAPDH

F

KRT8-1

| *Tg* | - | + | - | + |
| *Krt5* | +/+ | +/+ | -/- | -/- |

K8

K14

GAPDH

H

Mouse *Krt14* RNA - E18.5

Relative Fold Change

KRT8bc-1
KRT8-1

n.s.   *

Tg   -   +   +
Krt5   +/+   +/+   -/-

G

Relative Band Intensity

*   n.s.

● *Krt5* null
■ Replacement

KRT8bc-1   *KRT8-1*

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mice, while the markedly lower levels of K8 protein may have played a role in the failure to obtain successful rescues in *KRT8* replacement mice (see Discussion).

There is substantive evidence indicating interdependence in the steady-state levels of type I and type II keratin proteins in epithelial cells (Kulesh et al., 1989; Lersch et al., 1989). Accordingly, the levels of K5 protein are reduced in newborn $Krt14^{-/-}$ mouse skin (Lloyd et al., 1995), while the levels of K14 protein are reduced in newborn $Krt5^{-/-}$ mouse skin (Peters et al., 2001). In the *Krt5* null case, in particular, the levels of *Krt14* mRNA are also substantially reduced (Peters et al., 2001), hinting at the complexity of mechanisms regulating the balance between type I and II keratins in epidermal keratinocytes *in vivo*. We analyzed K14 expression as a surrogate marker for the "effective dosage" of type II keratin expression in the skin of E18.5 embryos. At the protein level, very weak K14 expression occurs in *Krt5* null embryos, as well as in *KRT8* replacement embryos (Fig. 2.2E, F), respectively emulating the complete absence of type II keratins and the low levels of K8 rescue protein observed in these settings. By comparison, and as expected, expression of K14 protein is stimulated ~2.7 fold in *KRT8bc* replacement embryos over *Krt5* null levels (Fig. 2.2G), consistent with the phenotypic rescue of viable *KRT8bc* replacement mice (Table 2.1).

Examination of *Krt14* mRNA levels in E18.5 embryos of relevant genotypes provides additional insight. Similar levels of *Krt14* mRNAs occur in *KRT8* and *KRT8bc* transgenic skin (with a normal *Krt5* locus), and these levels are slightly elevated relative to that seen in control embryos (albeit in a non-statistically significant fashion; Fig. 2.2H). *Krt14* mRNA levels are increased nearly 2-fold in the setting of *KRT8bc*

replacement embryos, exhibiting a striking and statistically-significant increase compared to *KRT8* replacement E18.5 embryos (Fig. 2.2H). These data clearly point to a mechanism(s) by which basal keratinocytes are able to sense the anomaly stemming from ectopically expressing other type II keratins, as well as sensing the difference between K8bc and K8 at the mRNA and/or protein level.

Analysis of keratin IFs in basal keratinocytes in primary culture and in the epidermis in situ. To further assess the behavior of the K8bc protein *in vivo*, control, *Krt5* null, and *KRT8bc* replacement E18.5 embryos were processed for skin keratinocyte isolation, primary culture and analysis via indirect immunofluorescence. In control cultures, K5 and K14 were co-localized (data not shown) and formed a classic pan-cytoplasmic array (Fig. 2.3A, A''), while K8 was typically absent (Fig. 2.3A') except in rare cells that may correspond to Merkel cells (data not shown), which express K8 naturally (Moll et al., 1984). In *Krt5* null keratinocyte cultures, no signal was detected for K5 and K8 antigens, as expected, while the signal for K14 was weak (Fig. 2.3C-C''). In *KRT8bc* replacement cultures, there was no signal for K5 (as expected), and K8bc and K14 were consistently co-localized in the context of normal-looking arrays of keratin IFs (Fig. 2.3D', D'' and data not shown). Images of control and *KRT8bc* replacement keratinocytes taken under identical exposure conditions did not exhibit reduced levels of K14 protein, in contrast to the western blot data presented in Fig. 2.2E. This may be due in part to the induced expression of other keratins in the context of primary culture of keratinocytes (Weiss et al., 1984; McGowan and Coulombe, 1998). These findings, obtained in mouse skin keratinocytes, significantly extend our previous work

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Figure 2.3: Analysis of Embryonic Skin Keratinocytes in Primary Culture by Indirect Immunofluorescence. Conventional fluorescence microscopy of keratinocytes harvested from $Krt5^{+/+}$ (control) (A-A''''), $Krt5^{-/-}$ ($Krt5$ null) (B-B''), or $KRT8bc^{Tg/-}Krt5^{-/-}$ ($KRT8bc$ replacement) (C-C'') E18.5 embryos (as indicated on top). Fixed cells were immunostained for keratin 5, keratin 8, and keratin 14 epitopes, as indicated at left. Nu, nucleus. Bars = 10μm.

Figure 2.3: Analysis of Embryonic Skin Keratinocytes in Primary Culture by Indirect Immunofluorescence.
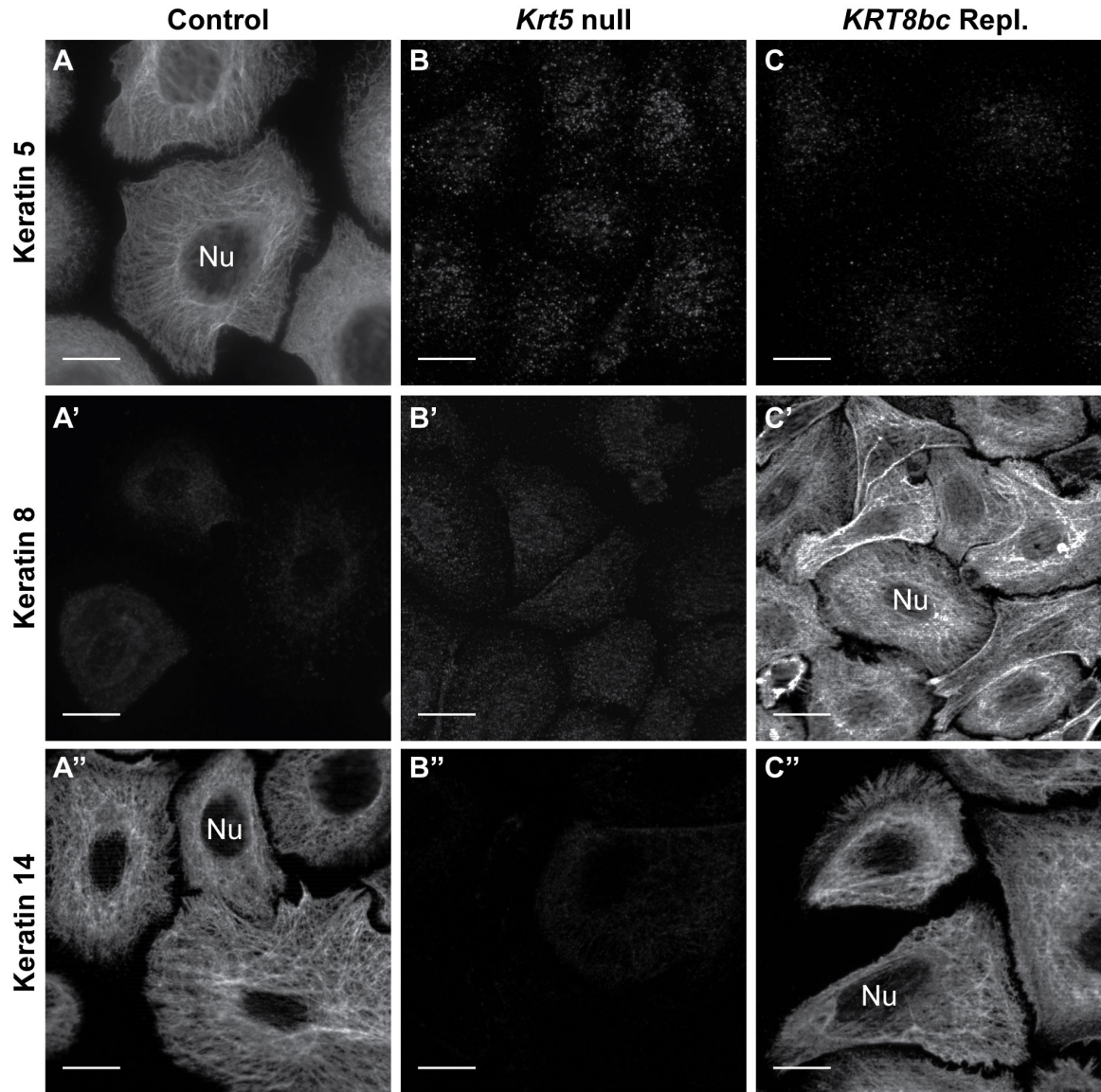
demonstrating that K8bc is competent for 10nm filament assembly and IF network formation when paired with type I K14 (Lee and Coulombe, 2009).

To further examine the innate features of basal keratinocytes including their keratin IF network, we processed E18.5 skin for routine transmission electron microscopy. At this late embryonic stage, the epidermis is mature, and adult-like barrier properties have been acquired (Lee and Coulombe, 2009). In this setting, basal keratinocytes are columnar in shape, with an ovoid nucleus oriented along the long axis of the cell (Fig. 2.4A-C). Hemidesmosomes and desmosomes are numerous and regularly spaced at the basal pole of basal keratinocytes and at sites of cell-cell contact, respectively, and exhibit association with electron-dense filamentous structures indicating attachment to keratin IFs (Fig. 2.4B, C). In addition, long bundles of keratin IFs oriented along the long axis of the cell can be readily be found on each side of the nucleus, and the mitochondria appear healthy (Fig. 2.4B,C). In areas of *Krt5* null embryonic skin for which the epidermis is intact, the overall thickness, stratification, and differentiated layers of the epidermis appear normal (Fig. 2.4D). That said, the basal keratinocytes are markedly flattened, and their nucleus is oriented parallel, rather than perpendicular, to the skin surface (Fig. 2.4D-F; Table 2.2). While desmosomes and hemidesmosomes persist, they do not appear to associate with electron-dense filaments; in fact, no keratin IF bundles can be detected anywhere in the cytoplasm of the basal cells (Fig. 2.4E, F). Virtually all mitochondria appear severely damaged in basal cells, as seen through their swelling, loss of cristae, and loss of matrix as reflected by the electron-lucent appearance (Fig. 2.4F, Table 2.2). The latter does not result from a fixation artifact, as mitochondria

Figure 2.4: Ultrastructural Analysis of E18.5 Epidermis *in situ*. $Krt5^{+/+}$ (control) (A-C), $Krt5^{-/-}$ (*Krt5* null) (D-F), and $KRT8bc^{Tg/-}Krt5^{-/-}$ (*KRT8bc* replacement) (G-I) mouse epidermis at E18.5 was analyzed by routine transmission electron microscopy of thin sections. Frames A, D, and G provide low-magnification surveys of the living layers of epidermis (basal, spinous, granular), while all other frames provide details of basal keratinocytes. kif, keratin intermediate filament bundles; mi, mitochondria; Nu, nucleus. Examples of hemidesmosomes and desmosomes are circled and boxed, respectively.

Figure 2.4: Ultrastructural Analysis of E18.5 Epidermis *in situ*.
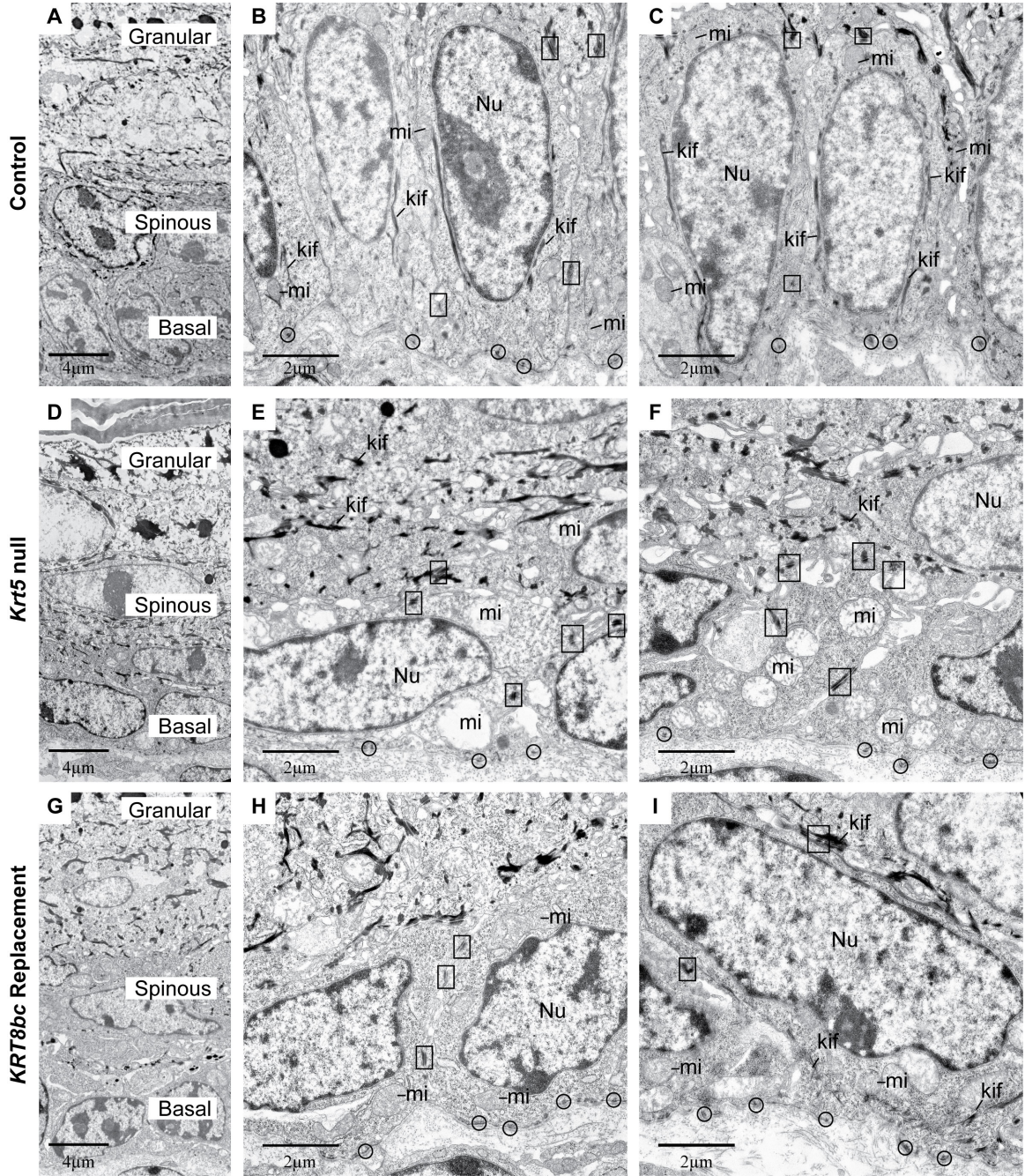
with normal size and ultrastructure can readily be found in epidermis-proximal dermal fibroblasts and in the upper layers of the epidermis (data not shown). Further, the ultrastructural features of the nuclei and cytoplasm clearly convey that basal keratinocytes are not undergoing apoptosis in intact skin of *Krt5* null E18.5 embryos (Lu et al., 2007). In addition to engendering acute tissue fragility, the genetic loss of *Krt5* leads to several major cytoarchitectural defects in the epidermis (see Table 2.2) that are largely confined to basal layer keratinocytes.

Several of these ultrastructural attributes are partially restored in basal keratinocytes of *KRT8bc* replacement E18.5 skin, correlating with a more mechanically sound tissue during harvesting and preparation (data not shown). Basal keratinocytes are cuboidal in shape, and their nuclei are consistently oriented perpendicular to the main axis of epidermis (Fig. 2.4G-I, Table 2.2). Interestingly, their nuclei still exhibit an aberrant contour, rather than the round or ovoid shape observed in control and *Krt5* null epidermis. The mitochondrial defects observed in *Krt5* null embryonic skin are also partially restored in the *KRT8bc* replacement skin (Table 2.2). Ultrastructural features including an electron-dense matrix and intact cristae are present in approximately half of observed mitochondria (Fig. 2.4H, I, and Table 2.2). Desmosomes and Hemidesmosomes appear normal, and are more likely to exhibit association with filament networks (Fig. 2.4H, I). Finally, electron-dense bundles of keratin IFs along the cellular periphery and in the general cytoplasm are observed (Fig. 2.4I). These bundles are smaller, however, and occur less frequently in *KRT8bc* replacement mice relative to

Table 2.2: Summary of Ultrastructural Observations Made in E18.5 (Pre-Birth) Epidermis.

Features	Control	*Krt5* null	*KRT8bc* Replacement
Suprabasal Ultrastructure	Normal	Normal	Normal
Hemidesmosomes (per cell)	4.9 ± 1.7	6.7 ± 3.0	5.5 ± 1.8
Desmosomes (per cell)	6.2 ± 1.4	4.6 ± 0.8	4.1 ± 1.6
Keratin filament bundles	Numerous	Absent	Occasional
Cell aspect ratio	1.58 ± 0.5	0.81 ± 0.4	1.08 ± 0.4
Nuclear aspect ratio	2.05 ± 0.9	0.66 ± 0.3	0.92 ± 0.5
Mitochondria (% normal)	98.8%	7.4%	52.9%

Control indicates *Krt5*$^{+/+}$, *Krt5* null indicates *Krt5*$^{-/-}$, and *KRT8bc* replacement indicates *KRT8bc*$^{Tg/-}$*Krt5*$^{-/-}$.

control epidermis (Table 2.2). A summary of the observed ultrastructural phenotypes, and their frequency, is presented in Table 2.2.

Adult KRT8bc *replacement animals exhibit several skin abnormalities.* KRT8bc replacement mice that survived to adulthood were indistinguishable from their littermates for the first several weeks after birth. To examine the status of the epidermis in these animals, tissue was harvested from a successful *KRT8bc* replacement adult and a control littermate, and processed for transmission electron microscopy. Analysis of these samples revealed a striking similarity between control and *KRT8bc* replacement epidermis. The shape and orientation of basal keratinocytes and the orientation of their nuclei appeared similar in both samples (Fig. 2.5). Keratin filament bundles were visible in the cytoplasm and near the nucleus, and were observed to be associated with hemidesmosomes and desmosomes in both samples (Fig. 2.5). Most notably, the mitochondria appear healthy in *KRT8bc* replacement epidermis, comparable to control epidermis (Fig. 2.5B, D). The most striking difference between the control and rescued skin is the persistence of the aberrant contour of the nuclei (Fig. 2.5B, D).

As the *KRT8bc* replacement mice aged, however, multiple phenotypes began to manifest. Most of the rescued mice developed alopecia starting as early as 6 weeks of age. Hair loss occurred consistently on the nape, and often progressed into larger hairless regions along the back, flanks, and occasionally on the face and other body areas (Fig. 2.6A). Additionally, all *KRT8bc* replacement animals developed severe ulcerative dermatitis on the ears and nape (Fig. 2.6D). These areas of ulcerative dermatitis did not respond to topical antibiotic ointment, and resulted in the formation of chronic lesions.

Figure 2.5: Ultrastructural Analysis of Adult Control and *KRT8bc* Replacement Epidermis *in situ*.
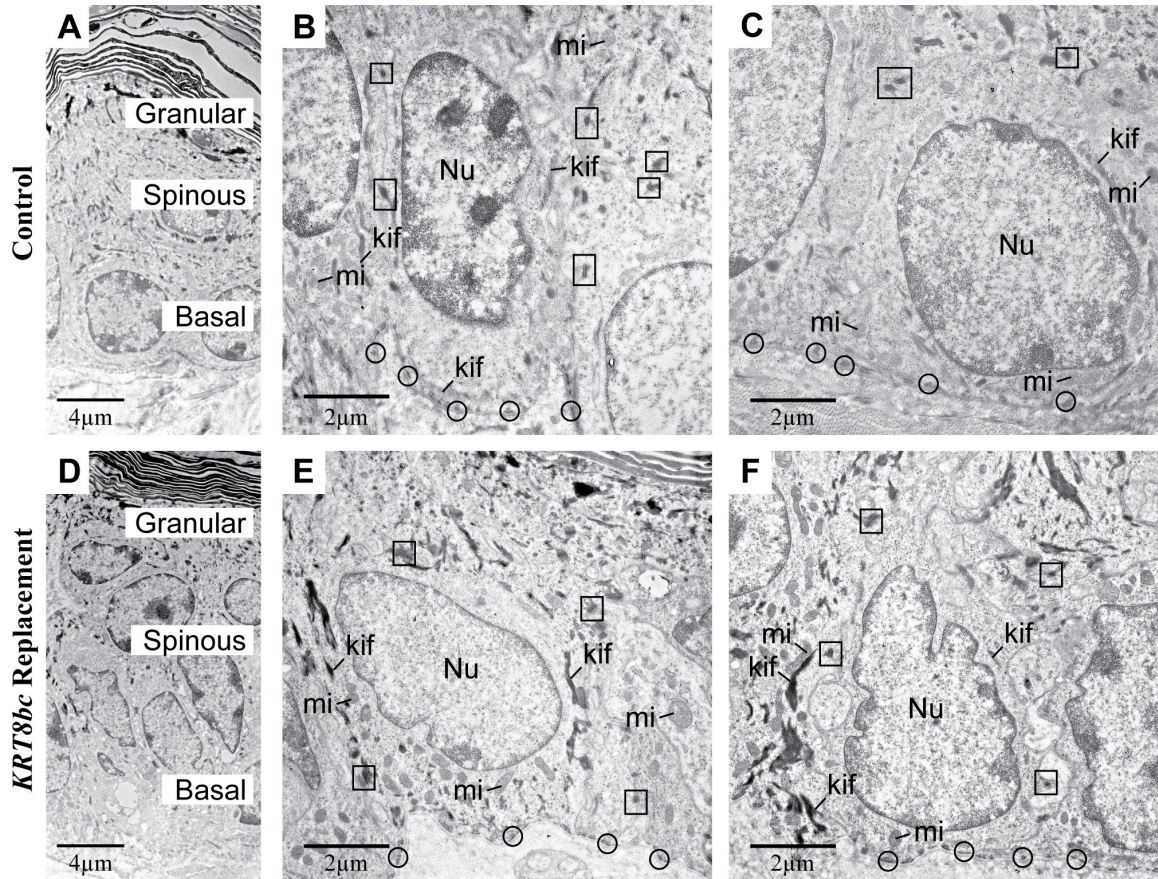
Krt5^{+/+} (control) (A-C), and *KRT8bc*^{Tg/-}*Krt5*^{-/-} (*KRT8bc* replacement) (D-F) epidermis in 11wk old mice by routine transmission electron microscopy of thin sections. Frames A and D provide low-magnification surveys of the living layers of epidermis (basal, spinous, granular), while frames B,C, E, and F provide details of basal keratinocytes. kif, keratin intermediate filament bundles; mi, mitochondria; Nu, nucleus. Examples of hemidesmosomes and desmosomes are circled and boxed, respectively.

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To further assess this phenomenon, routine histology was performed on normal-looking, hairy skin and on regions of affected skin. Hairy skin from *KRT8bc* replacement mice was comparable to normal control animal skin (data not shown), with a normal epidermis and telogen-stage hair follicles (Fig. 2.6B, E). Hairless regions of replacement animals showed an increased epidermal thickness compared to hairy skin (as expected), and enlarged sebaceous glands (Fig. 2.6C). Moreover, hair follicles were aberrantly shaped and oriented in hairless regions, and frequently appeared to be at the anagen stage of their cycle (Fig. 2.6C). In regions of lesional skin, the epidermis was markedly hyperplastic and the dermis was heavily inflamed (Fig. 2.6F). As with hairless skin, lesional skin contained hair follicles in anagen stage (when present), and were often improperly-oriented. Many hair follicles were without a visible hair shaft, and the associated sebaceous glands were greatly enlarged (Fig. 2.6F). Also of note was that the lesional skin contained numerous large cysts of pilosebaceous origin (data not shown).

DISCUSSION

In the current study, we set out to test the functional importance of the property of K5/K14 filament self-organization into cross-linked networks by targeting the expression of K8bc or wildtype K8 to the epidermis of *Krt5* null mice. K8bc is a chimera that is polymerization and self-organization competent when paired with K14, while K8 readily co-polymerizes with K14 but is unable to form cross-linked networks (Lee and Coulombe, 2009). We found that *KRT14* promoter-driven expression of K8bc rescued *Krt5* null mice from massive perinatal blistering and death with a 47% frequency, whereas expression of K8 was unable to afford any rescue in this setting. While the

Figure 2.6: Analysis of Late-Onset Phenotypes in Adult *KRT8bc* Replacement Animals. Macroscopic survey (A, D) and histology (B, C, E, F) illustrating the late-onset phenotype arising in *KRT8bc* replacement animals. A) Six month old adult *KRT8bc* replacement animals showing areas of both normal skin and areas of alopecia are shown. B) Hairy skin from *KRT8bc* replacement animal (see box "B" in frame A) showing normal histology. C) Phenotypic skin sample from *KRT8bc* replacement animal (see box "C" in frame A) showing misoriented, anagen-staged hair follicles and thickened epidermis. D) A 3 month old adult *KRT8bc* replacement animal showing both normal skin and areas of inflamed and hyperkeratotic, scaly skin. E) Hairy skin from *KRT8bc* replacement animal showing normal histology (see box "E" in frame D). F) Phenotypic skin from replacement animals (see box "F" in frame D) depicting hyperplastic epidermis, pilosebaceous cysts, and a high level of dermal infiltration suggesting an inflammatory and immune infiltration. Epi, epidermis; hf, hair follicle; sg, sebaceous gland. Bars= 100µm.

Figure 2.6: Analysis of Late-Onset Phenotypes in Adult *KRT8bc* Replacement Animals.
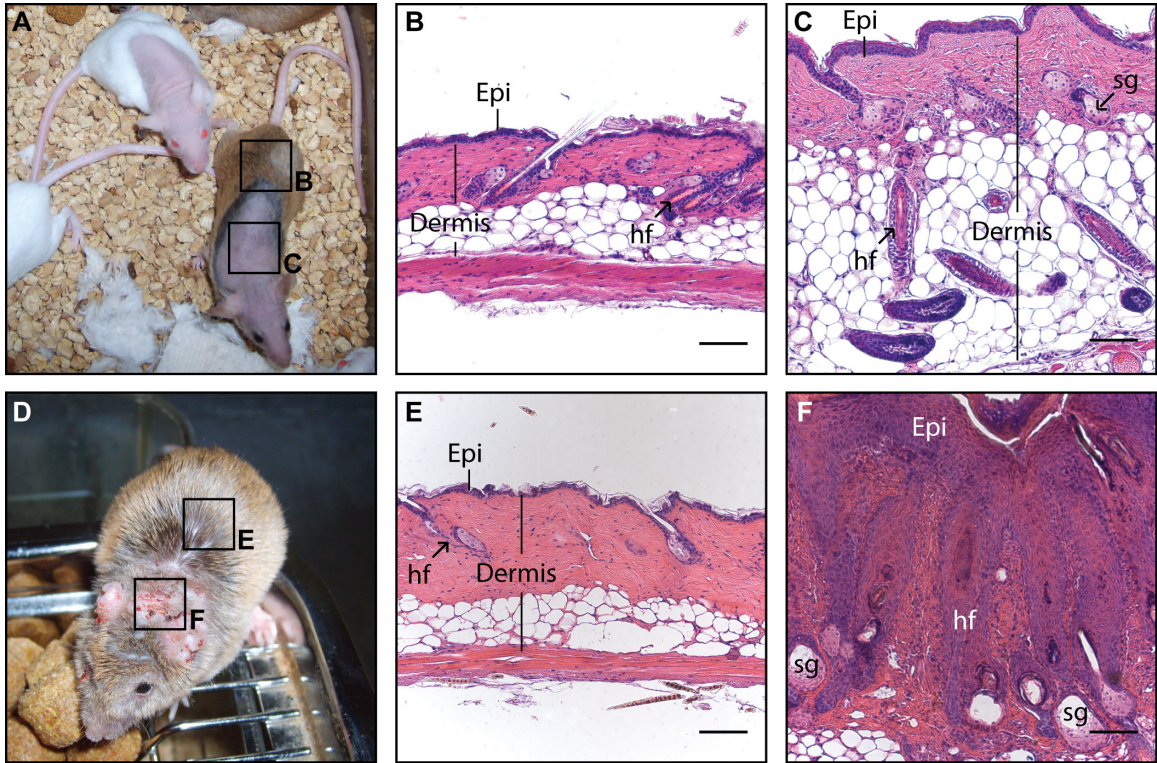
rescued *KRT8bc* replacement mice reached adulthood and were reproductively

competent, most eventually developed alopecia and all developed skin erosions; also,

their basal keratinocytes exhibited persisting ultrastructural anomalies (see Table 2.2).

These observations imply that, even when expressed at approximately half the levels of

endogenous K5 in control skin, the K8bc chimeric protein cannot fully support the

function(s) of K5 in mouse epidermis *in vivo*. Further, the occurrence of rescue from

overt cell and tissue fragility in neonatal and adult skin could be correlated with the

presence of keratin IF bundles in basal keratinocytes, consistent with (but not proving)

the hypothesis put forth in Lee and Coulombe (Lee and Coulombe, 2009).

In the first approximation, the significance of our protein replacement findings,

and of the differences observed between K8bc and K8, is mitigated by the relatively low,

and uneven, levels at which they were each expressed in *Krt5* null mouse epidermis.

However, recent studies suggest that much less than 50% of the normal (control)

complement of K5/K14 proteins suffices to rescue key phenotypic traits in keratin-free

keratinocytes in culture (Seltmann et al., 2013a; Seltmann et al., 2013b; Kroger et al.,

2013). Besides, our data suggest that the steady state levels of rescue transgene mRNA

differ depending on genotype (e.g. in transgenic mice vs. replacement mice) and the

rescue construct being expressed (e.g. K8 vs. K8bc), extending a previous observation of

striking context-dependent post-transcriptional regulation for ectopic keratin mRNAs in

basal keratinocytes (Paladini and Coulombe, 1999). While we cannot formally compare

the effectiveness of the K8 and K8bc proteins in replacing K5 in basal epidermal

keratinocytes, it is quite remarkable that sub-normal levels of K8bc (Fig. 2.2C), alongside

a partial rescue of endogenous K14 protein levels (Fig. 2.2E), were sufficient to effectively rescue skin blistering and death in nearly 50% of *KRT8bc* replacement newborns. Otherwise, the outcome of our study conveys that the property of 10nm filament formation, *in vitro* and *in vivo*, does not suffice to dictate a normal fate and/or function for an IF protein in basal keratinocytes of the epidermis, thereby significantly extending previous rescue efforts of a similar nature (Paladini and Coulombe, 1999; Kirfel et al., 2002).

Additionally, our *KRT8* transgenic mice did not feature a visible phenotype, likely the result of low expression levels, whereas Casanova et al. (Casanova et al., 2004) observed severe skin anomalies in transgenic mice that ectopically express wildtype K8 (at higher levels) in the epidermis. Our findings with *KRT8* transgenic mice are, in fact, similar to those reported by Kirfel et al. (Kirfel et al., 2002), who showed that ectopic desmin was able to form 10nm filaments in basal keratinocytes but could not rescue the *Krt5* null mice to any extent, correlating with low expression. In both the Kirfel et al. study and the current one, the data in hand precludes a formal assignment of the failure to rescue to the low prevailing levels of rescue protein expression achieved vs. abnormal protein properties in an ectopic setting.

Our ultrastructural assessment of late-stage embryonic *Krt5* null mice as well as adult *KRT8bc* replacement mice revealed novel and potentially important irregularities associated with K5 protein deficiency in mouse epidermis. Our findings corroborate the previous description of a complete lack of detectable keratin IFs in the cytoplasm of basal keratinocytes, and of largely normal-appearing suprabasal keratinocytes (Peters et al.,

2001). In addition to these observations, we also observed major cytoarchitectural

defects, e.g., aberrancies in cell shape and orientation, the shape and orientation of the

nucleus in basal keratinocytes, and major mitochondrial defects. At least in part, the

differences between our study and that of Peters et al. may be rooted in the use of

different protocols when preparing samples for analysis by transmission electron

microscopy (Peters et al., 2001). The marked alterations observed in the shape of basal

keratinocytes in *Krt5* null skin may alter their physical relationship with other key cell

types in the epidermis, including melanocytes and Langerhans cells. As such, they may

contribute to the aberrations observed in skin pigmentation (Betz et al., 2006) and

Langerhans cell density (Roth et al., 2009) in mouse models and/or individuals with

genetic alterations at the *Krt5* locus. In addition, our observations may well be related to

the findings of Lee et al. (Lee et al., 2012), who proposed that perinuclear K5/K14

filaments impart an oxidation-state dependent influence upon the size and shape of the

nucleus in newborn epidermis. On another front, the observation of a difference in the

number of desmosomal and hemidesmosomal plaques in *Krt5* null basal keratinocytes

(which are devoid of keratin IFs) is not surprising in light of similar findings obtained

when analyzing type II keratin-free keratinocytes (Kroger et al., 2013), and considering

the roles recently assigned to keratins in the localization and maintenance of desmosomes

(Seltmann et al., 2013b; Liovic et al., 2009).

The mitochondrial defects observed in late-stage embryonic *Krt5* null mice are

particularly interesting. The shape and position of mitochondria within cells has been

shown to be influenced by keratin IFs in a number of contexts. For example, we note that

similar mitochondrial defects have been reported in *Krt14* null mouse neonates (Lloyd et al., 1995), though they were shown to be associated with cytolysis, and therefore may have been a consequence of overt, post-lysis cellular distress. Also, we previously reported on the occurrence of intriguing electron dense inclusions in the mitochondrial matrix of epidermal keratinocytes from both *KRT16*-overexpressing transgenic mice (Takahashi et al., 1994) and *Krt16* null mice (Lessard et al., 2013). Additionally, a reduced mitochondrial size was reported in livers of *Krt8* null mice and in mice carrying the *KRT18 R89C* mutation (Tao et al., 2009; Kumemura et al., 2008), while Stone et al. (Stone et al., 2007) described an increased mitochondrial size in the skeletal muscle of *Krt19* null mice. Ongoing efforts in several laboratories, including our own, should shed light on the implications and mechanisms of K5's involvement in mitochondrial physiology and other important determinants of the structure and function of the epidermis and related epithelia.

ACKNOWLEDGMENTS

CHAPTER 3

ANALYSIS OF THE BIOLOGICAL FUNCTION OF NEWLY IDENTIFIED

KERATIN 5 POLYMORPHISMS WHICH IMPART INCREASED SUSCEPTIBILITY

TO BASAL CELL CARCINOMA

David M. Alvarado and Pierre A. Coulombe

INTRODUCTION

Keratins are intracellular fibrous heteropolymers that are expressed in epithelia of higher metazoans. While a primary function of keratin filaments is to provide structural support, the list of biological functions assigned to keratins is still expanding, and includes the modulation of cellular function through an impact on signaling pathways (Pan et al., 2013). Although these emerging functions play significant roles in the normal biological processes of the cell, keratins are also known to be involved in the pathology of several human diseases (Omary et al., 2009; Karantza, 2011; Pan et al., 2013). Cancer is one such disorder, and studies of a number of tumor settings are revealing potential roles for keratins in cancer. It has been determined that keratins 5, 6, and 17 are faithful markers of cancer of basal cell origin (Markey et al., 1992; Ordonez, 1998). In fact, expression of keratins 5 and 17 is shown to correlate with poorer patient prognosis in a number of cancer paradigms (van de Rijn et al., 2002; Karantza, 2011; Ide et al., 2012; Wang et al., 2013). Additionally, the aberrant expression of the simple epithelial keratin K8 in the skin induces severe dysplasia of the hair follicles and an increase in the progression of papillomas toward malignancies (Casanova et al., 2004). Given the recurring misregulation of keratins in cancers, as well as the known roles of keratins in signaling pathways, it is plausible that keratins are more than mere bystanders in this disease.

Basal cell carcinoma (BCC) is the most common cancer in patients of European descent. The majority of incidences of BCC occur sporadically, although BCC is one of

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the phenotypes associated with the hereditary disorder called basal-cell nevus syndrome (BCNS) (Gorlin, 1987; Epstein, 2008). BCNS, also known as Gorlin syndrome, can be caused by mutation in the *PTCH1* gene, a transmembrane receptor that inhibits hedgehog signal transduction in the absence of its ligand (Johnson et al., 1996). The discovery made in the context of Gorlin syndrome lead to the subsequent finding that the genetic and molecular basis of most cases of BCC in skin is aberrant Sonic hedgehog (Shh) signaling (Gailani et al., 1992; Hahn et al., 1996; Johnson et al., 1996; Epstein, 2008). Indeed, the overwhelming majority of sporadic BCC cases harbor mutations in *PTCH1*, while 10% of cases have mutations in the Shh downstream effector *SMO* that most likely render it resistant to inhibition by *PTCH1* (Aszterbaum et al., 1998; Xie et al., 1998; Epstein, 2008).

To examine the role of keratin 17 in the onset and progression of this disease, a mouse model with constitutive expression of a Shh transcriptional effector, Gli2, were crossed to a mouse strain harboring a null mutation at the *KRT17* locus (Depianto et al., 2010). In this setting, ear tumor onset was significantly delayed in the absence of keratin 17. This delay correlated with decreased cell proliferation along with major alterations in inflammation and immune response (Depianto et al., 2010). Specifically, the loss of keratin 17 caused a polarization of the immune response from a Th1/Th17-dominated to a Th2-dominated profile (Depianto et al., 2010). Such findings suggested that keratins are involved in immune modulation in the tumor setting, with an impact on tumor initiation and progression.

The type II keratin binding partner for keratin 17 in several cancer settings is keratin 5 (van de Rijn et al., 2002; Depianto et al., 2010). A recent genome-wide association study identified two single nucleotide polymorphisms (SNPs) that affect the coding region of keratin 5 and confer increased susceptibility to BCC (Stacey et al., 2009). These variants encode the following substitutions: glycine (Gly) to glutamic acid (Glu) at position 138 (referred to herein as K5 G138E) within the non-helical head domain, and aspartic acid (Asp) to glutamic acid (Glu) at position 197 (referred to herein as K5 D197E) within the central rod domain. These substitutions represent novel variants of keratin 5, in that they have not been previously identified as associated with human diseases, including Epidermolysis Bullosa Simplex (Stacey et al., 2009). While the process of tumorigenesis is a complex and multifactorial one, the molecular genetics of BCC and the known functions of keratin 5 point to several possible mechanisms by which these SNPs increase BCC susceptibility.

First, it is possible that these variants alter the keratin filament assembly, structure, and/or dynamics. While these variants have not been identified as a cause of EBS to date, Stacey et al. predicted that these substitutions would be deleterious (Stacey et al., 2009). EBS is caused by single substitutions in the coding region of *KRT5* in nearly 50% of all cases (Szeverenyi et al., 2008; Coulombe and Lee, 2012). There is some clinical evidence suggesting that these mutant proteins alter filament function in a subtle and sub-clinical fashion (Trufant et al., 2010; Shurman et al., 2006). One report described a transient EBS-like phenotype following the treatment with a retinoid for cutaneous T cell lymphoma (Trufant et al., 2010). Mutational analysis revealed that this

patient carried one allele encoding the K5 G138E variant (Trufant et al., 2010). At the proper dosage, retinoids attenuate epidermal keratin expression (Torma, 2011), and this may have led to K5 G138E attaining the stoichiometry needed to elicit a fragility phenotype. Another clinical report described a family with a mild form of EBS that presented with the unique co-morbidity of hyperkeratotic papules on the extremities of select offspring (Shurman et al., 2006). Mutational analysis of this family identified the EBS-causing mutation to be *KRT5 c.74C>T* (encoding the substitution P25L normally associated with a rare EBS subtype with pigmentation anomalies; see below), but the co-morbidity presented only in individuals carrying both this mutation and the allele encoding the K5 D197E variant (Shurman et al., 2006).

Another potential mechanism of increased BCC susceptibility by these keratin 5 variants is altered ultraviolet damage response. The most significant environmental risk factor for BCC is sunlight exposure; therefore, variants that affect the protection against, and repair of, UV-induced DNA damage repair could alter the susceptibility to develop BCC (Epstein, 2008). Consistent with this, variants of genes that regulate pigmentation in skin correlate with susceptibility to BCC (Han et al., 2006). A number of disease contexts have linked keratin 5 mutations with alterations in pigmentation (Gu and Coulombe, 2007). For example, EBS with mottled pigmentation (EBS-MP) (Uttam et al., 1996) and EBS Migratory (EBS-Migr) (Gu et al., 2003) are subtypes of EBS which presents with altered skin pigmentation in the form of hypo- or hyperpigmented patches (Fischer and Gedde-Dahl, 1979). Also, Dowling Degos Disease (DDD) is a non-EBS disease caused by haploinsufficiency of keratin 5 which also presents with aberrant

pigmentation (Dowling and Freudenthal, 1938; Betz et al., 2006). However, Stacey et al. (2009) found that body areas exposed to sunlight did not have more incidence of BCC than areas not exposed to sunlight (P=0.09), nor do these variants associate with any pigmentation trait in patients carrying the keratin 5 SNPs. While these data suggest that these variants modulate risk of BCC through mechanisms other than altered response to UV exposure (Stacey et al., 2009), this possibility deserves a deeper investigation.

A third possible mechanism by which the K5 G138E and K5 D197E variants could increase BCC susceptibility is via alterations in the emerging role of keratins as immune response modulators. As mentioned above, investigation of the role of keratin 17 in BCC has revealed immune response modulation as a potentially powerful mechanism by which keratins can influence tumor initiation and progression (Depianto et al., 2010). Genetic ablation of keratin 5 has also been shown to alter the immune profile in embryonic mouse skin, specifically increasing the expression of the cytokines Ccl2, Ccl19, and Ccl20 (see Table 3.2) (Roth et al., 2009). Therefore, one should consider the potential of these keratin 5 variants to impact the immunological landscape of the skin in such a way as to promote the cellular transformations leading to BCC.

Finally, another mechanistic possibility is a heretofore unidentified role for keratin 5 in modulation of Shh signaling. While there is no direct evidence for this, the number of signaling pathways modulated by keratins continues to increase steadily (see Chapter 1) (Pan et al., 2013). In the study presented in this Chapter, we continue the

analyses initiated by Andrew Sawaya (Sawaya, 2011) in an effort to determine the impact of these keratin 5 variants on cellular functions in HeLa cells.

EXPERIMENTAL PROCEDURES

Plasmids. Plasmids pEGFP-N1, pEGFP-N1-K5, pEGFP-N1-K5G138E, and pEGFP-N1-K5D197E were described previously (Sawaya, 2011). The open reading frame of wildtype *KRT5*, *KRT5* variant K5 G138E, and *KRT5* variant K5 D197E were PCR-amplified by the primers indicated in Table 3.1, digested with EcoRI and KpnI, and subcloned into pEGFP-C3 vector. The resulting plasmids were designated pEGFP-C3 (Vector), pEGFP-C3-K5 (wildtype *KRT5*), pEGFP-C3-K5G138E (designated *KRT5 G138E*), and pEGFP-C3-K5D197E (designated *KRT5 D197E*). Plasmid pEGFP-C3-K5E477K (designated *KRT5 E477K*), which expresses a mutated and EBS-causing keratin that severely disrupts keratin filaments (Gu and Coulombe, 2005), was generated by site-directed mutagenesis of the wildtype *KRT5* plasmid using QuikChange II KL Site-Directed Mutagenesis kit (Agilent Technologies) and the primers indicated in Table 3.1.

Cell lines and transfections. All transfection experiments were performed in HeLa cells (ATCC CCL-2) using GeneJuice® reagent (EMD Millipore) according to the manufacturer's instruction. Cells were maintained in low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin/Streptomycin. Cells were treated with 12-O-tetradecanoylphorbol-13-acetate

Table 3.1: List of Primers

Primer Direction	Primer Sequence (5'–3')	Comments
Forward	CCATGAATTCCAATGTCTCGCCAGTCAAGTGTGTCCTTCCGG	PCR Cloning (EcoRI site underlined)
Reverse	CCAAAGCGGTACCTTAGCTCTTGAAGCTCTTCCGGG	PCR Cloning (KpnI site underlined)
Forward	GCAAGCTGCTGGAGGGCAAGGAATGCAGACTCAGTGG	Site-Directed Mutagenesis E477K
Reverse	CCACTGAGTCTGCATTCCTTGCCCTCCAGCAGCTTGC	Site-Directed Mutagenesis E477K
Forward	CCAGGCTCTCAACTGAAGGC	For qPCR
Reverse	GAATCCCGTTCCGAGTGG	
Forward	CAGCCAGATGCAATCAATGCC	For qPCR
Reverse	TGGAATCCTGAACCCACTTCT	
Forward	GAAGGACACGGGCAGCAGAC	For qPCR
Reverse	GACACCGGGCTTGGAGCACTG	
Forward	GACTATGACACGACCACAGAGT	For qPCR
Reverse	TTCTGGCACGGAGTTGCATC	
Forward	GTGGCATTCAAGGAGTACCTC	For qPCR
Reverse	TGATGGCCTTCGATTCTGGATT	
Forward	AGCTGCGTTGCGTTTGTTTAC	For qPCR
Reverse	GCGAACACTTGCAGATTACTGA	
Forward	CCTGTCTTACTTTTCCGAAGGAC	For qPCR
Reverse	GGCTGGGCTAACATTGGATGA	
Forward	AGCGTGAGCCTGAATCTGTG	For qPCR
Reverse	ATCCCAACGGCAGTCAGTTTC	
Forward	TCTCAAAGCCTGTTCCACTCA	For qPCR
Reverse	TATGGCCTCGGCAAGTGTAAA	
Forward	TCGGTAACTGACTTGAATGTCCA	For qPCR
Reverse	TCGCTTCCCGTTTTAGCTGC	
Forward	ATGATGGCTTATTACAGTGGCAA	For qPCR
Reverse	GTCGGAGATTCGTAGCTGGA	
Forward	AATTCGGTACATCCTCGACGG	For qPCR
Reverse	TTGGAAGGTTCAGGTTGTTTTCT	
Forward	GGTGGGTGGTGAGATCAATGT	For qPCR
Reverse	CGCGGTTCAGTTCCTCTGTC	
Forward	GAAGAAGGTGCTAATGTCCTGAC	For qPCR
Reverse	GTCCCAGACTGTAATTTCGCC	
Forward	GGGGCACGAGTGATCTGTG	For qPCR
Reverse	GCATGATGTCTGGGTAACGCT	
Forward	ATTCTTGGTGGTCGCTAGGTA	For qPCR
Reverse	CGCCTTCTCCGATGTACTGC	

Table 3.2: Biological Relevance of qRT-PCR Targets

Gene	Gene Name	Comments
AIRE	Autoimmune Regulator	A transcriptional regulator that plays an important role in immunity by regulating the expression of autoantigens and negative selection of autoreactive T cells in the thymus.
CCL2	Chemokine (C-C motif) ligand 2	A pro-inflammatory cytokine which shows chemotactic activity for monocytes and basophiles. It has been implicated in the pathogenesis of such diseases as psoriasis, rheumatoid arthritis, and atherosclerosis.
CCL3	Chemokine (C-C motif) ligand 3	Also known as macrophage inflammatory protein 1 alpha. A small pro-inflammatory cytokine which recruits and activates polymorpho-nuclear leukocytes.
CCL19	Chemokine (C-C motif) ligand 19	This cytokine plays a role in normal lymphocyte recirculation and homing, and plays an important role in trafficking of T cells in thymus and in T cell and B cell migration to secondary lymphoid organs.
CCL20	Chemokine (C-C motif) ligand 20	Also known as macrophage inflammatory protein 3 alpha. A cytokine that is involved in chemotaxis of lymphocytes, and to a lesser extent neutrophiles.
CCR1	Chemokine (C-C motif) receptor 1	Receptor found on peripheral blood lymphocytes and monocytes. CCR1 is involved in signal transduction required for the recruitment of effector immune cells to the site of inflammation. Receptor for CCL3.
CXCL10	Chemokine (C-X-C motif) ligand 10	Ligand for the receptor CXCR3. Interaction with receptor results in stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.
CXCL5	Chemokine (C-X-C motif) ligand 5	Ligand for receptor CXCR2. Interaction with receptor results in recruitment of neutrophils, promotion of angiogenesis, and remodeling of connective tissue.
CXCR2	Chemokine (C-X-C motif) receptor 2	Also known as interleukin 8 receptor beta. This receptor mediates neutrophil migration to sites of inflammation. Receptor for IL8, CXCL5, and other CXC cytokines.
GLI1	GLI family zinc finger 1	A member of the Kruppel family of zinc finger proteins. The encoded transcription factor is activated by the sonic hedgehog signal transduction cascade and regulates stem cell proliferation.
HHIP	Hedgehog interacting protein	Hedgehog signaling is important in regulation of embryonic development. The protein encoded by this gene is a highly conserved, vertebrate-specific inhibitor of HH signaling.
IFNG	Interferon gamma	A member of the type II interferon family. The protein encoded is a soluble cytokine with antiviral, immunoregulatory and anti-tumor properties and is a potent activator of macrophages.
IL1B	Interleukin 1 beta	This cytokine is produced by activated macrophages. It is an important mediator of the inflammatory response, and is involved in modulating cell proliferation, differentiation, and apoptosis.

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IL6	Interleukin 6	Also known as interferon beta 2. A pro-inflammatory cytokine which is produced at sites of acute and chronic inflammation, and is involved in the maturation of B cells and fever production.
KRT17	Keratin 17	A type I intermediate filament protein expressed in epithelial append-ages and upregulated in response to wounds. Also upregulated in cancer, and shown to modulate the immune environment in cancer.
PTCH1	Patched 1	The receptor for the secreted ligand sonic hedgehog. Involved in the formation of embryonic structures and in tumorigenesis. Functions as a tumor supressor.
WNT2B	Wingless-type MMTV integration site family, member 2b	A secreted signaling factor which functions in a variety of processes including regulation of cell growth and differentiation, as well as human carcinogenesis.
WNT5A	Wingless-type MMTV integration site family, member 5a	A secreted signaling factor which is highly expressed in the dermal papilla of depilated skin. Involved in a variety of processes including regulation of cell growth, differentiation, and human carcinogenesis.

(TPA, Sigma) dissolved in DMSO or DMSO alone, added to complete media to a final concentration of 200 nM for 12h (Pan et al., 2011).

Protein analysis. For analysis of protein expression, urea-soluble proteins were extracted from transfected HeLa cells as previously described (Lee and Coulombe, 2009). Protein concentrations were determined with a Bradford assay kit (Bio-Rad). Equal quantities of protein were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and antibody incubations were performed in 5% milk in 1x TBS-T. Protein epitopes were detected with the following antibodies: rabbit anti-K5 AF-138, (Covance) and mouse anti- β-actin antibody (Sigma). Secondary antibodies used were horseradish peroxidase- conjugated goat anti-rabbit and goat anti-mouse. Blots were developed using the SuperSignal West Pico Chemiluminescent substrate kit (Bio-Rad) and were imaged with FluorChem® Q MultiImage III (Alpha Innotech). Band signal intensity was measured from these images with the AlphaView® Q software.

Immunofluorescence. HeLa cells were plated on coverslips and grown in DMEM supplemented with 10% FBS and Pen/Strep at 37°C and 5% CO_2. For analysis of filament networks, cells were transfected with the plasmids described above overnight with GeneJuice reagent according to manufacturer's instructions. Cells were allowed to grow for 72h, and then coverslips were fixed with ice-cold methanol at -20°C for 10 min. K5 was detected with rabbit anti-K5 AF-138 antibody (Covance). Secondary antibody used was goat anti-rabbit Alexa-594, and nuclei were stained with Hoechst fluorescent

dye. Images were taken on a Zeiss AxioObserver inverted microscope with an AxioCam

HRc camera. "Bundled" networks were quantified using ImageJ by counting the number

of cells with fluorescent signal above the maxima threshold of 60 per 40x frame. Cells

with "aggregated" keratins were counted manually, and cells with "distributed" networks

were determined by subtracting the bundled and aggregated cells from the total number

of transfected cells. Images were cropped and scale bars were added also using ImageJ.

Quantitative real-time PCR. Total RNA was isolated from HeLa cells using

RNEasy isolation kit (Qiagen) according to manufacturer's instructions. Complementary

DNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) according to

manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR®

Green Real-Time PCR Master Mix from Life Technologies on a Bio-Rad C1000™

thermocycler and CFX™ Real-Time System. Oligonucleotide primers used are defined

in Table 3.1. Delta Cq (ΔCq) was determined by subtracting the averaged Cq values for

Actin and GAPDH from the Cq of the target, and delta-delta Cq ($\Delta\Delta$Cq) for each

targetwas determined by subtracting the ΔCq of the control samples (DMSO-treated)

from the TPA-treated samples (Lessard et al., 2013). Relative fold change was calculated

by taking $2^{-\Delta\Delta Cq}$. Calculations were performed using Microsoft Excel.

Statistical analyses. All statistics were performed using Graphpad Prism

software. Two-way analyses of variance (ANOVA) were used to test for significance,

and adjusted P values are reported.

RESULTS

Mutant keratins form normal pan-cytoplasmic networks in HeLa cells. In a previous effort, purified recombinant wildtype and mutant keratin 5 proteins were co-assembled with keratin 17 *in vitro* and the resulting assemblies were assessed by electron microscopy (Sawaya, 2011). In this setting, the filaments formed by the variants K5 G138E and K5 D197E are not distinguishable from wildtype K5 (Sawaya, 2011). The filaments appear to be normal in diameter (~10 nm), and no obvious aberration in filament assembly or structure was evident (Sawaya, 2011). To assess the ability of the keratin 5 variants to integrate into preexisting keratin filament networks in epithelial cells, HeLa cells were transfected with wildtype *KRT5* or the *KRT5* variant cDNAs, and keratin filament networks were visualized by indirect immunofluorescence. This analysis revealed only subtle differences between WT K5 and K5 variants (Fig. 3.1). Each of the K5 proteins tested (WT K5, K5 G138E, and K5 D197E) readily integrated into the keratin IF networks, which in the majority of cases maintained their pan-cytoplasmic distribution and normal appearance (Fig. 3.1). For the purpose of quantitation, transfected cells were assessed based on their appearance and labeled as "distributed", "bundled", or "aggregated" (Fig. 3.2A). Analysis of over 1000 cells for each of the keratin 5 constructs tested revealed that the variants K5 G138E and K5 D179E were more likely to be associated with filament bundles relative to wildtype keratin 5 (Fig. 3.2B). This resulted in a statistically significant decrease in the so-called "dispersed" filament networks in HeLa cells transfected with cDNA encoding K5 G138E or K5 D197E, and a corresponding increase in "bundled" filament networks (Fig. 3.2B). None of the type II

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Figure 3.1: Observation of Keratin Filament Networks in HeLa Cells.
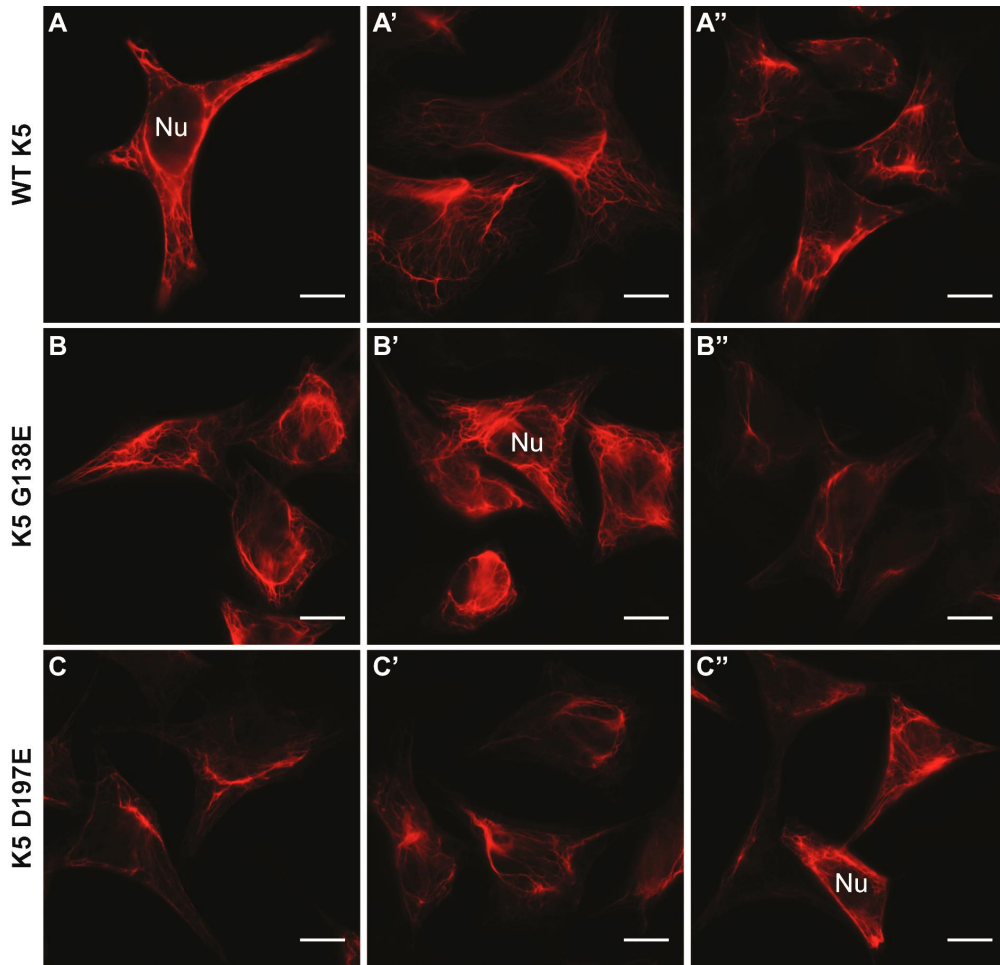
Figure 3.1: Observation of Keratin Filament Networks in HeLa Cells.

HeLa cells were transfected with cDNA encoding WT K5 (A-A"), K5 G138E (B-B"), or

K5 D197E (C-C"), and keratin filament networks were observed by indirect

immunofluorescence. In this setting, both keratin variants (B-C") properly integrated

into the endogenous keratin filament network of HeLa cells, and did not trigger any

obvious difference relative to networks containing wildtype K5 (A-A"). Nu, nucleus.

Bar=10μm

Figure 3.2: Analysis of Keratin Filament Networks in HeLa Cells.

A) Representative examples of each of the filament networks observed in HeLa cells transfected with cDNA encoding WT K5, K5 G138E, or K5 D197E. The examples of filament network phenotypes (dispersed, bundled, aggregated) shown are from cells transfected with wildtype *KRT5* cDNA. B) Frequency of filament network phenotypes in transfected HeLa cells. Cells transfected with cDNA encoding WT K5, K5 G138E, or K5 D197E were methanol-fixed and processed for indirect immunofluorescence. Filament networks were qualified as "bundled" if their fluorescent signal was above the maxima threshold of 60, as determined by the ImageJ software. "Aggregated" cells were counted manually, and cells with "distributed" networks were determined by subtracting "bundled" and "aggregated" cells from the total number of transfected cells. The percentages of each form of observed network is illustrated in graphical format. *, P=0.0001; **, P>0.0001. C) Steady-state protein levels of WT K5 (WT), K5 G138E (G138E), and K5 D197E (D197E) in transfected HeLa cells. Total protein was extracted from HeLa cells transfected with equal concentrations of cDNA and resolved by standard western blot analysis. ACTB protein was used as a loading control. Both K5 variants had higher steady-state protein levels when compared to wildtype K5.

Figure 3.2: Analysis of Keratin Filament Networks in HeLa Cells.
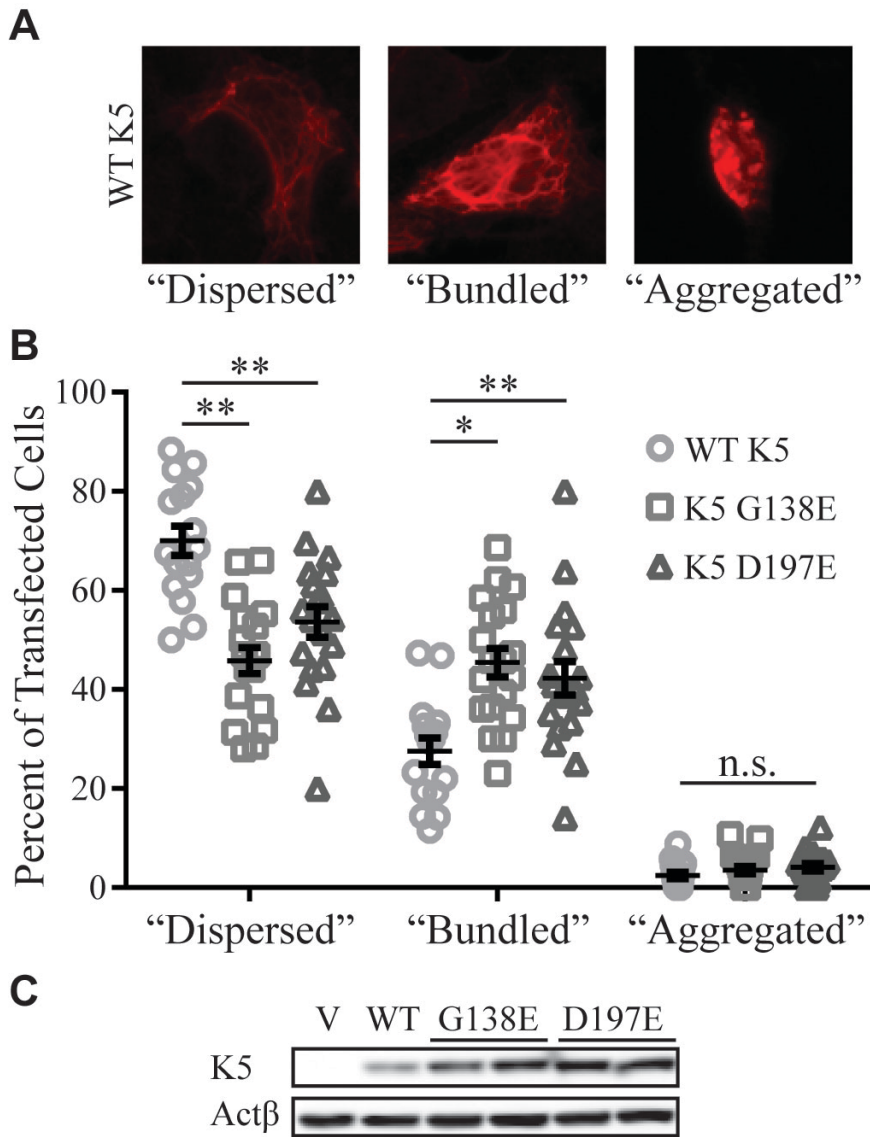
keratins assessed resulted in a significant increase in "aggregated" filament networks (Fig. 3.2B).

Since the characterization of keratin filaments as bundled networks is dependent on fluorescence signal intensity, one potential explanation for the significant increase in "bundled" networks in the *KRT5* variant transfectants is an increased amount of variant K5 protein content compared to WT K5 transfectants. To address this, the steady-state levels of keratin proteins were determined by extracting urea-soluble proteins from transfected HeLa cells and resolving by western blot (Fig. 3.2C). Using this analysis, the steady-state levels of variant K5 proteins were demonstrated to be higher than WT K5 despite the transfection of equal concentrations of cDNA (Fig. 3.2C). Whether this increased amount of variant K5 protein is the result of increased expression or sequence-related resistance to degradation remains unclear, and warrants follow-up analyses in the future.

To assess the impact of *KRT5* variant expression has on cellular signaling pathways relevant to BCC, transcript levels for specific genes (designated as "targets") was carried out using customized quantitative real-time PCR (qRT-PCR) arrays. Targets were selected from several paradigms, including some whose expression is elevated in known mouse tumor models (HPV[Tg], *GLI2*[Tg]) (Depianto et al., 2010), some determined to be differentially modulated in *Krt17*^{-/-} mice compared to *Krt17*^{+/+} mice (Depianto et al., 2010), and others found to be overexpressed in human BCC (Fig. 3.3a) (Bonifas et al., 2001; Katoh and Katoh, 2009). As an additional stimulus, transfected HeLa cells

Figure 3.3: Impact of K5 Variants on Cytokine Expression by qRT-PCR Analysis.

A) List of targets chosen for custom qRT-PCR analysis, and the setting in which they are upregulated or differentially regulated in a keratin- and/or cancer-specific manner (see Table 3.2 for associated biological significance). B) Targets that were unaffected by the expression of the two *KRT5* variants tested. Total RNA was queried with custom qRT-PCR arrays, and the relative fold change of TPA-treated cells over DMSO-treated cells is reported. The majority of targets assessed did not demonstrate a difference between wildtype and variant *KRT5* expression, including effectors of Shh and Wnt signaling pathways known to be markedly upregulated in BCC. C) Targets that demonstrated differential expression as a result of TPA treatment concomitant with the expression of K5 variants. Total RNA was queried with custom qRT-PCR arrays, and the relative fold change of TPA-treated cells over DMSO-treated cells is illustrated. The mRNA transcripts for genes *CCL2* and *CXCL5* exhibit distinct levels in cells expressing wildtype keratin 5 compared to cells expressing *KRT5* variants. *, P<0.01; **, P<0.001.

Figure 3.3: Impact of K5 Variants on Cytokine Expression by qRT-PCR Analysis.
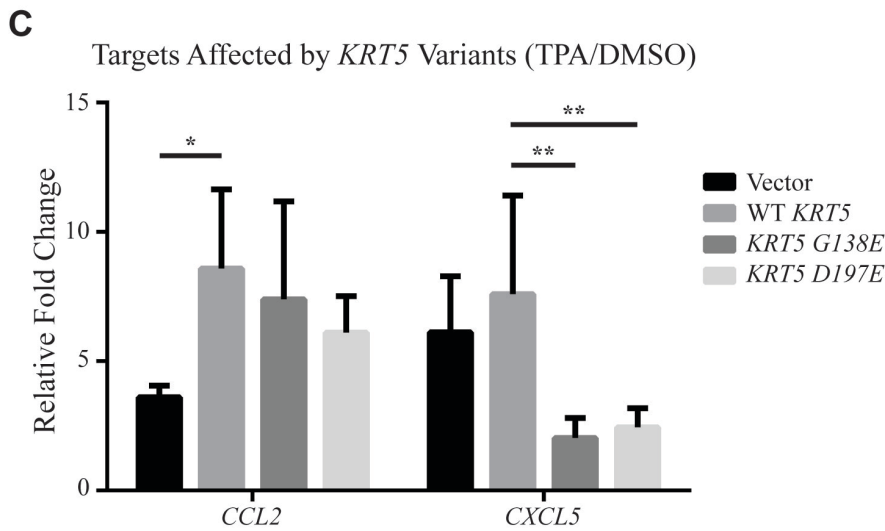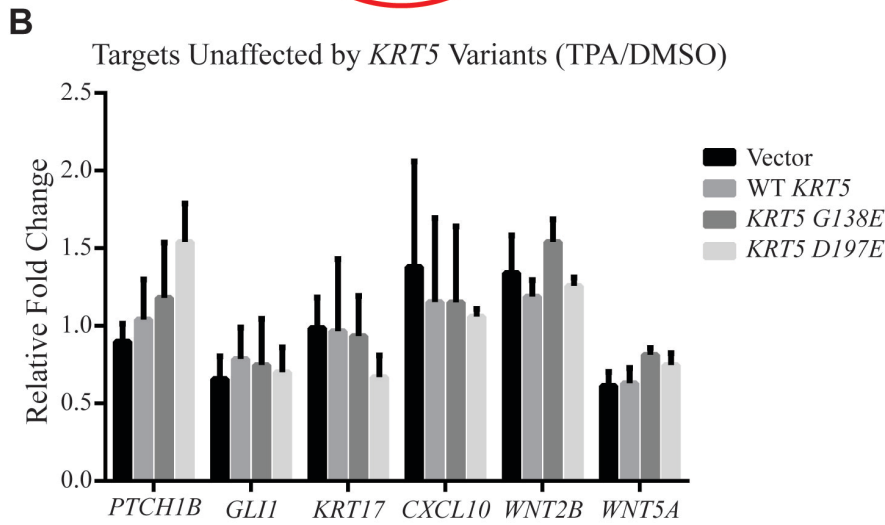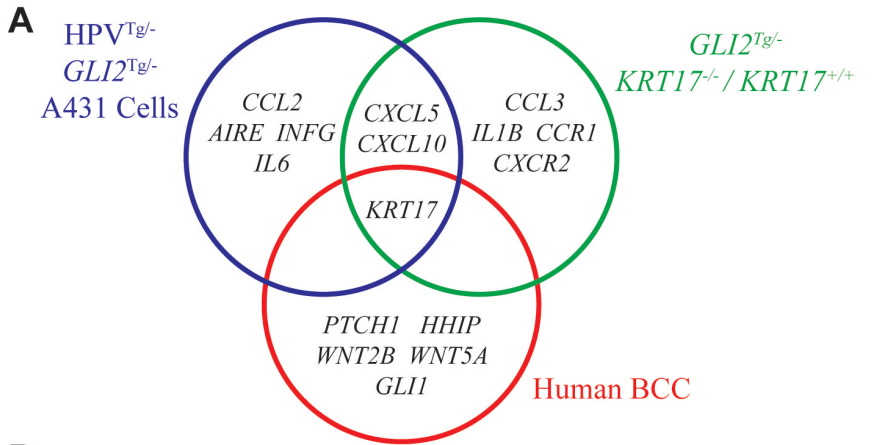
were treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) at a final concentration of 200nM. Using this method of analysis, we determined that the targets *AIRE, IL6, IFNG, IL1B, CCL3, CCR1, CXCR2,* and *HHIP* do not produce signal above levels detected in no reverse transcriptase control wells (data not shown), indicating they are either not expressed in HeLa cells, or below the levels of detection by this assay.

Of the remaining targets analyzed, the majority did not show significant differences between cells transfected with wildtype keratin 5 and cells transfected with keratin 5 variants (Fig. 3.3B). Included in this group are *PTCH1* and *GLI1*, two effectors in the Shh signaling pathway, and *WNT2B* and *WNT5A*, which are effectors of the Wnt signaling pathway that are upregulated in human BCC (Fig. 3.3A, B) (Yang et al., 2008). Also unaffected by the expression of the *KRT5* variants is their binding partner, *KRT17*, which is known to be upregulated in a variety of cancer paradigms (see Introduction; Fig. 3.3A, B).

Interestingly, two genes were found to be differentially regulated depending on the keratin variant being transfected. Specifically, *CCL2* was found to be upregulated as a result of TPA treatment in HeLa cells transfected with wildtype *KRT5* (Fig. 3.3C). Although the trend of increased *CCL2* expression is maintained in HeLa cells transfected with the *KRT5* variants K5 G138E and K5 D197E, the latter were determined to not be statistically significant relative to vector control (Fig. 3.3C). Another target, *CXCL5*, remained unchanged in HeLa cells transfected with wildtype *KRT5* when compared to

vector control; however, cells transfected with either *KRT5* variant displayed significantly reduced levels of *CXCL5* (Fig. 3.3C).

DISCUSSION

The current study set out to address whether two SNPs resulting in single amino acid substitutions in keratin 5 and associated with an increase risk in developing BCC (Stacey et al., 2009) modulate either the properties of keratin filaments or expression of specific genes in cultured HeLa cells. We found that, contrary to the predictions offered by Stacey et al., the K5 G138E variant (as well as the K5 D197E variant) did not impact keratin filament assembly or network formation in a deleterious fashion (Stacey et al., 2009). While the filament dynamics must be explored in more detail, the fact that both variants integrate into normal filament networks allows for the possibility that they impact BCC susceptibility through non-structural mechanisms.

Another possible mechanism by which keratin variants could impact BCC susceptibility is by interacting with specific signaling pathways in a manner differing from wildtype K5. It is still not clear whether keratins interact with these signaling pathways as small subunits or as filaments (Pan et al., 2013). Since both variants were associated with an increase in "bundled" networks, it is possible that they are not as readily available as subunits to interact with signaling pathways, relative to wildtype K5. Conversely, if IF-associating proteins with a role in signaling prefer a "bundled" moiety to bind to keratin intermediate filaments, the increase in such "bundled" networks in the presence of the keratin variants would likely augment such signaling cascades.

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The observation that expression of these variants affects the expression of *CCL2* and *CXCL5* differently than wildtype *KRT5* provides the possibility of immune modulation as the mechanism by which these keratin variants influence BCC risk. Interestingly, these variants did not increase the expression of their binding partner, *KRT17* (see Fig. 3.3), which is normally expressed in HeLa cells (Moll et al., 1982). These data provide evidence suggesting that keratin 5 exhibits immunomodulatory behavior independent of that observed with K17 (Depianto et al., 2010). Overall, the findings of this preliminary study provide a number of promising avenues to pursue in future studies.

ACKNOWLEDGMENTS

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

David M. Alvarado and Pierre A. Coulombe

The work presented in this dissertation addresses several outstanding questions in epithelial biology. In Chapter 2, we presented strong evidence supporting the importance of keratin filament self-organization towards their structural support function *in vivo*. We replaced the endogenous keratin 5 with a bundling-competent chimeric keratin, *KRT8bc*, and the resulting replacement animals survived to adulthood with a 47% frequency. This finding is quite remarkable, given that greater than 30% of the wildtype human K8 coding sequence is replaced by the corresponding coding sequence of human K5 in the *KRT8bc* chimera. For instance, this substitution far exceeds the threshold required to cause EBS in the human population, where often only a single amino acid substitution is necessary for the presentation of disease (Coulombe and Lee, 2012). We also demonstrated that this chimeric keratin becomes part of filament bundles (albeit to a lesser degree than K5) in basal keratinocytes of the epidermis in adult replacement animals. Finally, we uncovered a previously unidentified role of keratin 5 in the maintenance of cellular architecture and the integrity of mitochondria. Both of these new phenotypic traits were ameliorated by the expression of *KRT8bc* in the epidermis of keratin 5 null mice.

Despite the production and characterization of quite a large number of transgenic founders and lines, the highest expression levels achieved for a replacement protein, namely K8bc, were at ~50% of endogenous keratin 5 levels prevailing in wildtype skin. This fact precluded a direct comparison between the *KRT8bc* and *KRT8* transgenes. Because of this, future studies exploring the functions of keratin chimeras would be best served to use a knock-in strategy in lieu of a transgenic approach. Nonetheless, the

partial rescue of keratin 5 null mice with *KRT8bc* represents a significant finding that warrants further study. For example, in unaffected skin of adult replacement mice, the mitochondria exhibited a healthier morphology, and filament bundles were apparent. However, mitochondrial status was not assessed in the phenotypic skin of *KRT8bc* replacement mice. One clinical study revealed that hair and skin abnormalities can be indicators of underlying mitochondrial disorders (Bodemer et al., 1999). The role of thyroid hormones in the regulation and maintenance of the epidermis, especially of epidermal mitochondria, is coming to light (Tiede et al., 2010; Paus, 2010; Vidali et al., 2014). In fact, thyroid hormones have been shown to influence keratin expression in hair follicle stem cells (Tiede et al., 2010). Another recent study proposed that mitochondrial biogenesis is necessary for the proper development of hair follicles (Vidali et al., 2014). It is therefore possible that one explanation of the alopecia observed in adult replacement animals is reflecting the incomplete rescue of the mitochondrial phenotype observed at E18.5. This emerging keratin-mitochondrial link is an area which would warrant further investigation.

Also of interest was the observed disruption in the cellular architecture in *KRT5* null E18.5 animals. Previous studies have suggested a role for keratins 5 and 14 in regulation of basal cell architecture (see Chapter 1). A recent study revealed that a trans-dimer, homotypic disulfide bond involving residue Cysteine 367 in K14 impacts nuclear size and shape in early differentiating epidermal keratinocytes (Lee et al., 2012). Therefore, the aberrant cell and nuclear shape is likely the result of the keratin deficiency in basal cells of *KRT5* null animals. This altered cell shape and aberrant nuclear contour

potentially alters their physical relationship with other key cell types in the epidermis, including melanocytes and Langerhans cells. As such, these observations offer an explanation for the differences in skin pigmentation (Betz et al., 2006) and Langerhans cell density (Roth et al., 2009) seen in mouse models and/or individuals with genetic alterations at the *Krt5* locus. The persisting nuclear contour defect observed in *KRT8bc* replacement embryos may also potentially explain the ulcerative dermatitis that presents in all replacement animals. Therefore, the underlying cause of this phenotype also warrants further inquiry. Since the genetic ablation of keratin 5 results in increased Langerhans cell infiltration within the epidermis (Roth et al., 2009), it is possible that either the genetic ablation of K5 or the expression of K8bc is promoting a pro-inflammatory environment within the mouse epidermis. Therefore, the immune status of adult *KRT8bc* replacement mice should be explored in more detail.

In Chapter 3, we presented an initial analysis of the effects that two *KRT5* variants have on keratin filament networks and specific target mRNA expression in HeLa cells. We found that, relative to WT K5, the K5 G138E and K5 D197E variants generated detectable differences in filament organization, steady-state protein levels, and cytokine expression. However, these findings need to be reproduced and expanded, and otherwise there are still many unanswered questions in this line of investigation. For example, it is unclear whether the increase in "bundled" networks is a real phenomenon, or rather a consequence of higher steady-state levels of variant protein on average in transfected cells. To further distinguish these two possibilities, protein stability experiments should be undertaken. Also, additional information on the filament dynamics would be garnered

by high- and low-speed sedimentation experiments using *in vitro* filament assemblies. This has been attempted previously, though the results were unclear (Sawaya, 2011). Also, examination of high-salt protein extracts prepared from transfected cells would shed further light on the potential differences in solubility between these keratin variants.

A recently developed reagent that could prove useful in the examination of these *KRT5* variants is the global type II keratin knockout mouse model (Vijayaraj et al., 2009; Seltmann et al., 2013b). Indeed, reintroduction of a single type II keratin results in the re-formation of keratin filament networks comprised of a single type I and type II keratin pair (Seltmann et al., 2013b). Using such keratin-free cells will allow for a clear readout of differences in the keratin filament networks comprised of keratin 5 variant proteins. Analysis of these cells might also sharpen our understanding of the immunomodulatory effects specific to these variants in a more biologically relevant context.

Finally, it is important to note that the process of tumorigenesis is not one that can be easily replicated using short-term expression of these keratin variants in a cell culture setting. In fact, the impact that these variants have on BCC risk in patients is modest (combined odds ratio of 1.35), and manifest their effects over much of the patient's lifetime (Stacey et al., 2009). To assess the impact these keratin variants would have on a model organism over its lifetime, a knock-in approach should be used. This model would allow investigators to observe the effect of the variants as either heterozygous or as homozygous alleles. It would also be possible to subject these animals to a variety of

external stimuli pertinent to BCC, including ultraviolet radiation, and compare wildtype

mice to mice expressing variants of *KRT5*.

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Curriculum Vitae

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Education

2007-2014   Johns Hopkins School of Medicine, Baltimore MD
             Cellular and Molecular Medicine Graduate Training Program
             Ph.D. Cellular and Molecular Medicine *(anticipated March 2014)*
             Ph.D. Thesis: Assessing the contribution of self-organization of keratin
             intermediate filaments to their biological functions *in vivo*.

2005-2007   University of South Florida, Tampa FL
             College of Arts and Sciences, Honors College
             Undergraduate Student
             B.S. Microbiology, *magna cum laude*
             Honors Thesis: The *BRCA* mutation *185delAG* increases maspin
             expression in human ovarian surface epithelial cells.

2002-2005   Hillsborough Community College, Brandon FL
             Undergraduate Student
             A.A. Premedical, *with high honors*

Research Experience

May 2008-   Johns Hopkins Bloomberg School of Public Health, Baltimore MD
Present   Department of Biochemistry and Molecular Biology
             Thesis Research, Laboratory of Dr. Pierre A. Coulombe
             In my thesis research, I have developed and characterized eight transgenic
             mouse lines and used these lines with a keratin knockout mouse to
             generate protein replacements mice to explore the biological function of
             keratins *in vivo*. Techniques included molecular cloning, mouse
             husbandry and timed matings, microsurgery, isolation of keratinocytes for
             primary culture, immunofluorescence, protein purification, Western
             blotting, transmission electron microscopy, conventional PCR and
             quantitative RT-PCR, and data analysis.

Oct 2007–   Johns Hopkins School of Medicine, Baltimore MD
May 2008   Cellular and Molecular Medicine Graduate Training Program
             Graduate Student Rotations

             Fall: Dr. Fred Bunz, Department of Radiation Oncology

             Winter: Dr. Pierre A. Coulombe, Department of Cell Biology

             Spring: Dr. Ben Ho Park, Department of Oncology

Sept 2006–   University of South Florida, Tampa FL
May 2007   College of Medicine Pathology and Cell Biology
             Undergraduate Research, Laboratory of Dr. Patricia Kruk
             I explored the impact of the mutant protein BRCA 185delAG (BRAt) on
             the production of the serine protease maspin in cultured human ovarian
             epithelial cells. It was determined that BRAt protein expression increases
             the levels of both maspin RNA and protein. Also, since maspin has anti-
             tumor capabilities, transfection with either BRAt or maspin cDNA
             resulted in a decrease in cell proliferation following chemotherapeutic
             treatments. This work could be applied to determining future
             chemotherapeutic techniques. Techniques included cell culture, RT-PCR
             and Western blotting.

2006   Massachusetts Institute of Technology, Cambridge MA
             MIT Summer Research Program, Department of Biology
             Undergraduate Research, Laboratory of Dr. Anthony Sinskey
             I studied the shift in electrophoretic mobility of a megaplasmid maintained
             in the bacterium *Streptomyces padanus* MITKK-103, a newly isolated
             Actinomycin X_2 producer. The shift in mobility was found to correlate
             with the culture of the bacteria in media which allowed for the production
             of this DNA intercalating agent. My work suggested the cause of the
             change in mobility was the intercalation of Actinomycin X_2 during the
             preparation of the samples. Techniques used included bacterial culture
             and pulsed-field gel electrophoresis.

Teaching Experience

2012-2013   Towson University, Towson MD
             Adjunct Professor, BIOL120: Principles of Biology
             Developed syllabus and taught course materials to non-science major
             undergraduate students for two semesters. Experience included
             completion of a Teaching Pedagogy seminar lead by the class coordinator.

2008-2009   Johns Hopkins School of Medicine, Baltimore MD
Tutor, Graduate-level courses in <u>Genetics</u> and <u>Pathways and Regulation</u>

2003-2005   Hillsborough Community College, Brandon FL
Supplemental Instruction Leader, <u>Introduction to Computers and Technology</u>, <u>College Chemistry 1</u>, <u>Organic Chemistry 1</u>, and <u>Introduction to Anthropology</u>

Publications

Alvarado D.M., Coulombe P.A. (2014) Directed Expression of a Chimeric Type II Keratin Partially Rescues Keratin 5 Null Mice. *Manuscript under review.*

Poster Presentations

2010   "Is Self-Mediated Organization of Keratin Filaments Required for their Structural Support Function *in vivo*?" **D. Alvarado**, C.H. Lee, P.A. Coulombe. *Presenter*, Gordon Research Conference on Intermediate Filaments, Tilton NH

2007   "BRCA1 *185delAG* Mutation Increases Maspin Expression in Human Ovarian Surface Epithelial Cells" **D. Alvarado**, J. O'Donnell, N. Johnson, P. Kruk. *Presenter*, USF Undergraduate Research Symposium, Tampa FL

2007   "Behavior Analysis of a Megaplasmid Found in *Streptomyces padanus* MITKK-103" **D. Alvarado**, K. Kurosawa, P. Lessard, A. Sinskey. *Presenter*, USF Health Science Center Student Research Day, Tampa FL

2007   "BRCA1 185delAG Mutant Protein (BRAt) Induces Maspin Expression in Human Ovarian Surface Epithelial Cells" J. O'Donnell, N. Johnson, **D. Alvarado**, P. Kruk. American Association for Cancer Research, Los Angeles CA

2006   "Behavior Analysis of a Megaplasmid Found in *Streptomyces padanus* MITKK-103" **D. Alvarado**, K. Kurosawa, P. Lessard, A. Sinskey. *Presenter*, Annual Biomedical Research Conference for Minority Students, Anaheim, CA

Conference Participation

2010   Gordon Research Conference on Intermediate Filaments.
Tilton NH

2006   Annual Biomedical Research Conference for Minority Students.
Anaheim CA

Awards and Honors

2010	Carl Storm Underrepresented Minority Fellowship, Intermediate Filaments GRC
2007-2009	NIH NIGMS T32 Training Program in Cellular and Molecular Medicine (GM08752)
2007	Highest Achievement, MARC U-STAR Scholars Program
2006	Anne and Werner von Rosenstiel Undergraduate Research Symposium Award
2006	Outstanding Poster Presentation, USF Health Science Center Student Research Day
2005-2007	NIH Minority Access to Research Careers Undergraduate Student Training in Academic Research (MARC U-STAR) Program Participant
2005-2007	USF Honors College Scholarship
2005-2007	Latino Scholars Transfer Scholarship
2004-2005	Who's Who Among Students in American Junior Colleges
2004-2005	Academic Excellence in Chemistry
2004-2005	Outstanding Leadership in Supplemental Instruction
2004-2005	Outstanding Leadership in Student Government Association
2003-2005	Dean's List, Hillsborough Community College
2003-2004	Outstanding Service in Supplemental Instruction
2003-2004	Cynthia Mansour Mathematics Scholarship

Leadership Activities and Committee Participation

2009-2014   <u>Chemical Safety Officer</u>, Laboratory of Dr. Pierre A. Coulombe. Baltimore, MD
Ensured the safety of lab members by being responsible for training in the use of Personal Protection Equipment and hazardous materials, and was also in charge of proper collection and disposal of hazardous materials. With my leadership, our lab successfully passed every annual inspection with zero violations.

2005	<u>Student Co-Chair</u>, Student Activity and Service Fee Budget Committee.
	Tampa FL
	Along with Faculty Co-Chair, presided over meetings to determine distribution of ~$2.5 million designated for student activities.
2004-2005	<u>Assistant Jurisprudence</u>, Florida Junior/Community College Student Government Association District 3.
	District 3 FL
	Assured the student-written constitution was upheld during all official meetings and events; Served as council member during a constitutional violation hearing.
2004-2005	<u>President</u>, Hillsborough Community College Brandon Campus Student Government Association.
	Brandon, FL
	Presided over general meetings, during which money was allocated for various student-oriented events on campus; Served as liaison between students and faculty.
2004	<u>Vice President</u>, Hillsborough Community College Technology Club.
	Brandon, FL
	Played active role in planning and implementation of student-oriented events, including presenting a request for fund matching through the Student Government Organization for a technology showcase and luau directed towards the evening student body.

Volunteer Activities

2012	Student Sight Saver's Program and Bienestar: Assisted in translating during free eye examinations for underserved Hispanic population. Baltimore, MD
2004, 2005	Paint Your Heart Out; Tampa FL
2004, 2005	American Cancer Society Relay for Life; Seffner FL
2005	Metropolitan Ministries, Tampa FL
2005	"Senior Prom for Seniors," Active participant in recreational event in a home for senior citizens; Brandon FL
2004	"Wildlife Rehabilitation Center," Painting and repairs after hurricane damage; Tampa FL
2004	"November 2," Non-partisan voter awareness and assistance program; Tampa FL