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REVIEW

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Multiple roles for keratin intermediate filaments in the regulation of epithelial barrier function and apico-basal polarity

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ABSTRACT

As multicellular organisms evolved a family of cytoskeletal proteins, the keratins (types I and II) expressed in epithelial cells diversified in more than 20 genes in vertebrates. There is no question that keratin filaments confer mechanical stiffness to cells. However, such a number of genes can hardly be explained by evolutionary advantages in mechanical features. The use of transgenic mouse models has revealed unexpected functional relationships between keratin intermediate filaments and intracellular signaling. Accordingly, loss of keratins or mutations in keratins that cause or predispose to human diseases, result in increased sensitivity to apoptosis, regulation of innate immunity, permeabilization of tight junctions, and mistargeting of apical proteins in different epithelia. Precise mechanistic explanations for these phenomena are still lacking. However, immobilization of membrane or cytoplasmic proteins, including chaperones, on intermediate filaments ("scaffolding") appear as common molecular mechanisms and may explain the need for so many different keratin genes in vertebrates.

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Epithelial barriers, cytoplasmic intermediate filaments and innate immunity co-evolved with multicellularity in metazoans

Epithelial barriers represent the earliest tissues organized in metazoans.[1,2] The early development of cell-cell contacts sealing the paracellular route and polarized distribution of membrane proteins[3] and cytoskeleton[4] are characteristic of epithelia and common to all metazoan. From a functional standpoint, epithelial barriers provided early metazoans with the evolutionary advantage of defining an internal milieu, different from external sea or fresh water, and digestive cavity (e.g. in Cnidarians) enabling them to catch and digest bigger prey.[4]

Along with the increased complexity of the multicellular organisms, various molecular mechanisms and cellular structures co-evolved in metazoans. In this review, we will address the role of cytoplasmic intermediate filaments (IF),[5] and specifically keratins (K), which diverged from an ancestral lamin at the origin of metazoan lineages.[6] We will focus on the poorly recognized relationship between keratins and atypical PKC (aPKC), a component of the PARtition defective (Par) proteins or Par complex, which is essential for the acquisition of apico-basal polarity in epithelia,[7] first discovered in *C. elegans*.[8] We will review evidence indicating a more general role of keratins in protection from chemically-induced apoptosis, and regulation of other signaling pathways. Finally, larger, more complex, multicellular organisms also faced the challenge of attacks by single-celled organisms and viruses. Accordingly, innate immunity pathways evolved along with multicellularity[9] as a defense. A growing body of evidence suggest that keratin IF, and innate immunity interact in vertebrate epithelial barriers. Some of these interactions, which are possibly based on ancient evolutionary advantages for barrier function, are involved in pathophysiological mechanisms in human disease.

Keratins, mechanical is not mechanistic

Cytoplasmic IF are represented by keratins (type I and II) in epithelia. So far, 28 keratin genes (Krt) have been identified in the human genome, not counting

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the hair keratins. Keratin proteins (K) are obligate heterodimers of type I (13 genes) and type II (15 genes). All keratins display a central rod domain, involved in dimerization, and globular head and tail domains.[5] Filament formation requires, minimally, the expression of one type I and one type II keratin genes. Usually, more than two keratin genes are transcribed. In the adult, the hepatocytes are the archetypal example where only one type I (K8) and one type II (K18) are expressed.[10,11] Table 1 schematically summarizes the expression of type I and II keratins in tissues mentioned in this review. For a comprehensive description of keratin expression patterns see.[11,12]

As it is the case for other IF proteins, keratins are subjected to several post-translational modifications including phosphorylation, glycosylation, acetylation and sumoylation. These modifications control assembly / disassembly of the filaments, function, and subcellular distribution (reviewed in.[13]).

There are many differences in the biology of IF as compared with better-known microtubules and actin. IF are not polarized and there are no molecular motors using IF as substrates. Importantly, there are no IF in common model organisms such as yeast and flies, which yielded an early wealth of knowledge in the tubulin and actin fields. In addition, the insolubility of IF makes co-immunoprecipitation very difficult. Furthermore, with the exception of withaferin A,[14] drugs specifically affecting IF are lacking. Efforts to find drugs that correct the effects of keratin mutations by high-throughput screens are currently under way [15] and may yield novel pharmacological tools for keratin IF.

Because of the difficulties indicated above, knockout mouse models and knockdown of keratins in tissue culture cell lines have been the major molecular approaches available for functional studies of IF. Even those approaches are not straightforward. Knockout models are often embryonic lethal, such as the K8 knockout mouse in the C57B1/6 background (94% penetrance, [16]) or the pan-keratin II knockout mouse.[17] In other cases, keratin deficient mice showed very subtle phenotypes, possibly because of the keratin redundancy, for example, the K7 knockout.[18]

The mechanical function of keratin IF is textbook knowledge. It is self-evident in the epidermis, as well as in the mechanical characteristics of isolated keratin IF in vitro.[19] Epidermolysis bullosa simplex (EBS) is the paradigm of a mechanical disorder caused by mutations in K5, K14,[20] and in a few cases plectin, a keratin linker protein.[21] Shear stress on the epidermis causes blistering in these patients. Some features of the K14 mutations phenotype, however, seem to suggest that there are possible non-mechanical mechanisms involved. The R125P K14 mutation elicits JNK signaling.[22] Mutant K14 increases TNFα secretion and sensitivity.[23] Those observations were recently highlighted by findings of increased caspase 8 both in lesional and non-lesional areas in EBS patients[24] which suggests the mutations have additional consequences beyond a simple break of cells. Moreover, evidence from the Magin lab supports the notion that expression of the R125P K14 alone makes cells mechanically weaker than cells lacking keratins altogether.[25] Furthermore, data from Marceau lab suggest that in internal epithelia, keratins 8/18 contribute to cell stiffness via cortical actin, by activation of the ROCK signaling pathway.[26] This suggests that even mechanical properties may be partially explained by a signaling function. Finally, to help maintain the barrier together, keratins attach to desmosomes, which represent a critically important intercellular junction, and provide adhesive force to keep epithelial cells together. This will be reviewed in a separate section.

Conversely, extensive analysis of various keratin knockout models has shown that IF play non-mechanical functions, providing epithelial cells with protection against stress not related to deformation due to external forces (e.g., chemical stress). In the following sections we will review some of the consequences of loss, mutation, or overexpression of keratins in epithelial barriers. They comprise highly interlinked effects on apoptosis or survival, innate immunity, intracellular signaling, and apico-basal polarity.

Table 1. Keratin expression in tissues described in this review.

Epithelium	Cell type	Type I	Type II	References
Simple	Hepatocytes	K18	K8	12
	Intestine crypt	K18 K19 K20 K23	K8 K7	131
	Intestine villus	K18 K19 K20	K8	95
	Pancreatic ducts	K18 K19	K4 K8 K7	132
Stratified	Epidermis basal	K14 K15 K17	K5 K6	11
	Epidermis suprabasal / spinous	K16 K10	K6 K1 K2*	11
	Mammary gland duct	K14 K17 K18 K19	K5 K7 K8	11

Note. *Epidermis spinous/granular

Keratins protect liver, placenta, and skin epithelia from apoptosis

The first piece of evidence linking keratins with protection of epithelia from apoptosis came from the K8 knockout mouse. The high mid-gestational mortality in these animals is due to apoptosis in the liver and the placenta, more specifically, in the giant trophoblast cell layer. More importantly, the defect could be rescued by TNFα-deficient mothers or TNFR2-null offspring.[27,28] In fact, K8 or K18 deficient cells were found to be two orders of magnitude more sensitive to TNFα-induced apoptosis.[27] K18 provides resistance to Fas-mediated liver failure, but not through common apoptotic mechanisms.[29] These early surprising findings were reproduced in other systems as well. K17 null mice showed apoptosis in hair matrix cells,[30] which is also TNFα-dependent.[31] Lack of one allele of K14 also results in hypersensitivity to TNFα, keratinocyte apoptosis, and Naegeli-Franceschetti-Jadassohn syndrome.[32] In summary, it is important to highlight that in all these cases the stress is TNFα (that is "chemical stress"), not mechanical.

It seems appropriate to emphasize that the effect of keratin deficiency on apoptosis is different in the intestinal epithelium, as compared to the liver or the skin. No increases in the rate of apoptosis or necrosis were observed in K8 null small intestine enterocytes.[33] Moreover, a paradoxical resistance to apoptosis, which seems to be dependent on microbiota, was found in colonocytes in the same animal model,[34] suggesting tissue-specificity for this function of IF.

Not only keratin knockout, but also mutations result in changes in susceptibility to apoptosis. Overexpression of K18 R89C mutant predisposes hepatocytes to Fas- but not TNF-mediated apoptosis.[35] Conversely, in epidermis, expression of K10/14 chimeras increase predisposition to skin cancer by suppression of apoptosis.[36]

The role of keratins in the protection of epithelial cells from apoptosis has translational significance. Exonic mutations in K8 and 18 predispose to liver chemical injury. Patients with severe amoxicillin-clavulanate, isoniazid or nitrofurantoin drug induced liver injury showed specific keratin mutations,[37] acute liver failure,[38] and primary biliary cirrhosis.[39] It is not surprising that keratin mutations predispose to injury in the liver: Unlike in other epithelia, there is no keratin redundancy in hepatocytes cells.

Molecular mechanisms involved in IF-mediated epithelial protection

Mechanistic studies of the roles of keratin IF in epithelial cell survival are far from complete. The broadly accepted interpretation of the available evidence is that IF, which represent abundant insoluble structures, provide a solid surface to bind and immobilize proteins which would be otherwise soluble in the cytosol. This phenomenon is generally referred to as "scaffolding." IF scaffolding sequesters several proteins away from the locations where they should fulfill their functions such as the cytosol, the inner surface of the plasma membrane, or the vicinity of specific receptors. It is generally assumed that proteins attached to the IF scaffold are not functional. However, in the case of Hsp70 chaperones (discussed below), we have found that scaffolding modifies or even enhances function. For the anti-apoptotic function of keratin IF, several proapoptotic proteins were found attached to the IF scaffold and released upon specific signaling. In the absence of IF, such as in K deficient mouse models, the same proteins would be readily available in the cytosol. IF scaffolding was shown for TRADD,[40] c-Flip,[41] DEDD,[42] caspases,[43] and Pirh2, a RING-H2-type ubiquitin E3 ligase.[44] Other, as yet not fully understood mechanisms include a switch to a FasR-mediated apoptosis and possible disruption of lipid rafts.[45]

Although no specific evidence currently supports possible synergy among these mechanisms, in theory, they are not mutually excluding. The molecular details of protein binding to keratins are also unclear. Direct interactions with keratin domains have been shown for proteins such as TRADD to the K18 and 14 head domain.[40] On the other hand, many keratin binding proteins have been discovered, including chaperones,[46-49] plectin (epiplakin 1), a cytoskeletal linker,[50] and desmosomal proteins [51] among others, which greatly increase the number of potential binding sites to indirectly attach proteins to the keratin IF. Accordingly, the protein-protein interactions involved in the IF scaffold are complex and far from fully understood.

Keratins in protein chaperoning

Early studies found several chaperones associated to the IF scaffold: Hsp70 isoforms [48] are tightly attached to keratin IF.[52] Likewise some members of the Hsp40 family bind stably to the C-terminal region of K18.[46]

Small cochaperones, such as Bag1 also bind to the IF scaffold under pro-inflammatory upregulation.[53] Filensin IF bind α-crystallin.[54] Hsp74 is directly attached to K1 in the urinary bladder epithelium,[55] and Hsp27 interacts with keratin tetramers.[56]

Because the IF scaffold is generally thought to be a sink that prevents proteins from carrying their normal function (e.g. TRADD, mentioned above), the first question that comes to mind is whether or not keratin-bound chaperones are functional. When purified keratin IF containing Hsp70 and Hsp40 are used in a standard luciferase refolding assay to measure Hsp70 chaperoning activity, they can refold chemically denatured luciferase at a similar rate as soluble (cytosolic) Hsp70.[52,53]

By subcellular fractionation, it was determined that Hsp70/40 chaperones exist in both soluble (cytosolic) and IF-bound forms. The latter represents approximately 10% of the total cellular chaperone in epithelial cells in culture.[52] This apparently modest fraction epitomizes an emerging question about the quantitative significance of the IF scaffold. For any of the proteins attached to the IF scaffold, is immobilization on IF sufficient to affect overall cellular function? Multiple independent pieces of evidence seem to be required to answer this question. There is at least one example of an Hsp70/40 substrate ("client") which fulfills multiple criteria leading to the conclusion that IF-associated chaperoning can exclusively refold some proteins which the cytosolic counterpart does not process. PKCs (including "atypical" aPKC) are refolded (rescued) by Hsp70 chaperones which bind a conserved site on PKC partially overlapping the turn motif[57] (reviewed in[58]). The steady-state levels of aPKC are deeply decreased in K8 deficient enterocytes that is in cells where loss of IF is complete (no redundant type II keratin) and increased in K8 transgenic overexpressers.[52] Likewise, the half-life of aPKC is decreased nearly 7-fold in cultured epithelial cells under K8 knock-down. In that case, there are no transcriptional or translational changes in the expression of the protein.[52] In vitro, after subcellular fractionation, cytosolic extracts lacking IF, which maintain full luciferase refolding capacity, failed to refold aPKC. Conversely, keratin IF also active in luciferase refolding were capable of aPKC refolding when supplemented with PDK1, the kinase that stabilizes aPKC active conformation.[59] In summary, the keratin scaffold with its associated proteins is necessary and sufficient to carry out aPKC refolding.

The presence of chaperones on the IF scaffold may have three possible functions. First, they are involved in chaperoning keratins themselves [60-62] and, accordingly, also associated with misfolded keratin aggregates, such as Mallory-Denk bodies in alcoholic and non-alcoholic steatohepatitis.[63] Second, IF attached chaperones may bind misfolded, inactive proteins, thus enhancing the binding capacity of the IF scaffold to peptides that would not bind directly to keratins. Examples of tight binding of not fully folded proteins to keratin IF have been reported.[64] However, the role of chaperones and the functional effects of this type of scaffolding sink remain unclear: In each case the fate of proteins attached to the IF scaffold needs to be established. The third type of function includes active chaperoning of specific proteins, epitomized by aPKC. It is uncertain how many other proteins may require specific folding at the IF scaffold. Data on steady-state protein levels of a group of kinases, including Akt, which are known to be clients of Hsp70/40, showed changes under K8 knockdown. Therefore, there is an indication that other kinases, in addition to aPKC, may be dependent on IF.[52] Accordingly, it is possible that a keratin/Hsp70–40 complex may regulate other signaling pathways through kinase stability.

Keratins in signaling

The effects of keratin expression on signaling pathways are among the most intriguing features of IF. Quite possibly, it is one of the central functions of IF in the regulation of epithelial barriers. Yet, it is one that remains very poorly understood. Examples of profound signaling changes induced by keratin deficiency or mutations abound and are summarized in Table 2.

Some of the signaling pathways affected by keratin loss or mutations are also involved in the development and maintenance of tight junctions (TJ). That is the case of aPKC in the PAR "polarity complex."[65-67] Likewise, ERK1/2 controls expression of TJ proteins,[68] and junction formation.[69,70] Furthermore, JNK has been shown to be part of a pathway that modulates trans-epithelial resistance [71] and ZO-1 assembly.[72] Loss of K76 results in increased skin permeability via loss of claudin 1.[73] Finally, IF-dependent changes in intracellular signaling are consistent with increases in epithelial Dextran 3000 permeability [52] in the intestine. In brief, changes in signaling

Table 2. Examples of effects of changes in keratin expression or keratin mutations on signaling pathways.

Affected keratin(s)	Effects	Ref.
K19 knockdown	Enhanced Akt signaling (decreased PTEN)	133
K19 knockdown	Destabilization of HER2 / decreased ERK	134
K5 and 14 mutations	Epidermolysis bullosa simplex (EBS) rescued by ERK inhibition	135
K17 overexpression	Activates Akt signaling in Ewing sarcoma	136
K17 overexpression	Activation of transcriptional regulator AIRE	106,137
K17 knockdown	Decreased pTyr-23 annexin A2	138
K14 knockdown (and decreased partner K5)	Decreased pAkt and enhanced Notch1	139
K14 overexpression	Increased JNK-MAPK signals	22
K8/K18 or K8/K19 overexpression	Raf-1 is released from 14-3-3 by stress	80
K8 null hepatocytes	Fas-activated apoptosis mediated by DEDD	140
K8 null hepatocytes	Inactive p38 MAPK, p44/42 MAPK and JNK1/2 are released from IF upon activation during apoptosis	141
K8 knockdown	Increased PI3K/Akt activation	142
K8 knockdown	Protein kinase C, cell adhesion and migration	45
K8 null mouse and K8 knockdown	Post-translational downregulation of aPKC via Hsp/Hsc70	33,52
K18 knockdown	MacroD1 (LRP16) retention in the cytoplasm	143
K17 knockout	Increased TNFR – NF-kB activity through TRADD	31
Global type I or II keratin knockout: rescue by expression of K6/K17 or K5/K14	Increased PKCα activity, desmosome destabilization	92
Global type II keratin knockout	GLUT1 – 3 mislocalization, AMPK and mTOR activation	113
Global type II keratin knockout	Increased EGFR and PKCα-dependent Erk1/2 signaling	112
Global type II keratin knockout	Rack1-keratin interactions modulate PKC-α signaling	144

Note. Bolded protein names indicate evidence for binding to keratin IF (scaffolding). Knockdown refers to RNAi manipulation in cell lines. Overexpression indicates vector-mediated transcription in cell lines.

pathways represent poorly studied links between keratin IF and epithelial barrier function.

The mechanistic details of how keratin defects or mutations modulate cellular signaling are still unclear and will need additional investigation. A few hypotheses, such as those that follow, may have to be tested for each specific signaling effect of keratin.

14-3-3 proteins bind phospho-peptides to sequester kinases or their substrates (reviewed by [74]). 14-3-3ζ, ε and σ have been shown to bind keratin IF.[75-78] 14-3-3 proteins bind keratins through their phosphorylated sites.[75] However, 14-3-3 are dimers with two aligned phosphorylated domain binding sites on the sides of a central channel. Accordingly, 14-3-3 dimers can scaffold two different proteins,[79] or, in this case bring another phosphoprotein to the IF scaffold. An example of this mechanism is the attachment of the Raf-1 kinase to the IF scaffold, mediated by 14-3-3. Under oxidative stress, Raf-1 phosphorylates keratin and is released from the scaffold.[80]

Mechanotransduction. Keratinocytes are under frequent and potentially strong mechanical stress. Mechanotransduction involves calcium influx as well as phosphorylation of epidermal growth factor receptor, and ERK1/2.[81](reviewed by [82]). In lung-derived cells, more subtle shear stress induces changes in IF that depend on aPKC activation.[83] Accordingly, defects in mechanical properties of these cells due to keratin deficiency may induce signaling changes. The role of mechanical stress and mechanotransduction is more difficult to conceptualize in cells subjected to comparatively very minimal mechanical stress, such as hepatocytes or epithelial cells in culture. Nonetheless, studies of how changes in mechanical properties of the keratin-deficient cells may result in further downstream changes in PKC, Akt, ERK, or JNK-MAPK signaling seem to be warranted. At this time, effects of mechanotransduction on other signaling effects cannot be ruled out for most of the consequences of keratin deficiency reviewed here.

Chaperoning. In a previous section we already discussed how keratin-associated chaperones maintain steady-state levels of aPKC. Whether or not similar keratin-dependent mechanisms are involved in maintaining the normal folding of other kinases, or preventing their degradation, remains a testable hypothesis.

In summary, there is extensive experimental evidence supporting the notion that a group of pro-survival and

stress-response signaling pathways require normal keratin IF. Possible mechanistic explanations for this requirement are multiple and additional data will be necessary to identify how and to what extent keratins affect specific kinase (or phosphatase) activities. In the next sections we will discuss the role of keratin IF in specific signaling pathways involved in desmosome assembly and innate immunity response.

Keratins and intercellular bonds in epithelial barriers: Is desmosome function dependent on if expression?

Keratin IF are anchored to the cell surface at desmosomes mediating attachment to the neighboring cells through desmocollin and desmoglein.[51] Keratin IF also connect to hemidesmosomes, which attach epithelial cells to the extracellular matrix at the basal membrane through integrin $\beta 4$.[84] There is no question that desmosomes and hemidesmosomes are important for epithelial barrier integrity. For example, in the skin and mucosae, autoantibodies against desmosome (desmogleins in pemphigous) and hemidesmosome (in pemphigoid) surface proteins, are associated with autoimmune blistering disease.[85] Mutations in desmosomal proteins result in arrhythmogenic cardiomyopathies and in skin syndromes (reviewed by[86]). The "Ogna type" epidermolysis bullosa simplex is caused by a missense mutation in plectin, which links keratin IF to the hemidesmosome plaque.[87]

Because of the tight association of IF to desmosomes and hemidesmosomes, it seems natural to ask whether or not filamentous keratins are necessary for the assembly and function of these junctions. Published evidence suggest mixed answers to this question.

K8-null embryonic cells[88] and K18-null hepatocytes[89] lacking IF still, display desmosomal plaques with normal ultrastructure, except, of course, for the absence of filaments. This is consistent with the current model of desmosomal assembly, which involves e-cadherin induced recruitment of desmoplakin and desmogleins, localized activation of PKCα followed by IF attachment at a late phase (reviewed by[84]). Conversely, keratin null keratinocytes display scattered plectin and hemidesmosome components, along with faster cell migration,[90] suggesting that keratin binding is necessary for hemidesmosome plaques to coalesce. This view of desmosome formation independent of IF, however, was recently challenged by evidence showing that specific deletion of K1/K10 (skin "differentiated" suprabasal keratins) results in smaller desmosomes with decreased amounts of desmoplakin and desmocollin, but normal plakoglobin.[91] This intriguing dependence of desmosome structure on specific keratin types, was recently clarified by data from Magin lab using global type I or type II keratin cluster knockout mouse keratinocytes, rescued by lentiviral-mediated expression of either K14 or K17. The resulting keratinocytes express all the keratins of the non-targeted cluster, and are rescued by lentiviral-mediated expression of one keratin of the knockout cluster. Cells expressing K14/K5 pairs displayed normal desmosomes. On the other hand, cells expressing K17/K6 pairs showed fragmented, less-stable desmosomes. Interestingly, the difference does not seem to be related to mechanical anchoring of the desmosome plaque to the filaments, but rather to the ability of K5/K14 IF to maintain PKCα away from the plasma membrane, possibly in an inactive conformation.[92] This is, therefore, another instance where the signaling function of the filaments is mechanistically prevalent over mechanical interactions.

Keratins in innate immunity and inflammation

Like for other signaling events, there is a growing body of publications reporting multiple roles of keratin IF in the regulation of innate immunity and epithelial inflammatory response. While these functions are typically associated with cells of myeloid lineage, epithelial barriers respond to infection or chemical stress by activating primitive innate immunity, primarily but not exclusively, via the NF-kB pathway. The results of this response include partial opening of TJ with loss of barrier function, secretion of anti-bacterial proteins, and recruitment of macrophages and other immune cells via cytokines (reviewed by [93]). Keratin-deficient cells and keratin mutations predisposing to inflammation reveal that IF play an important role in the regulation of the epithelial innate immunity responses in the skin and the intestinal epithelial barriers. Some examples are summarized in Table 3.

The paradigm of anti-inflammatory effects of keratins is the K8 knockout mouse. In the C57B1/6 background it is embryonic lethal,[16] but partially viable, i.e. submendelian proportions of pups are born in the FVB/N background.[94] In these animals, IF are fully abrogated in hepatocytes and the villus

Table 3. Examples of effects of keratin loss or keratin mutations on inflammatory mechanisms.

Affected keratin(s)	Effects	Ref.
K16 knockout	Regulates innate immunity in response to epithelial barrier opening	99
R156H K10 overexpression	Activation of p38, secretion of TNFα and RANTES	109
K17 knockout	Polarizes immune response, Th2 cytokine profile	145
K5 knockout	Transcriptional upregulation of pro-inflammatory cytokines IL-6 and IL-1β	108
K8 knockout	Th2 chronic intestinal inflammation	96
K8 / 18 mutations	Intestinal cell barrier function	98
K8 / 18 knockdown	Activates NF-kB in cancer cells	142
K10 expression in basal layer of epidermis	Decreased NF-kB activity	100

epithelium in the small intestine, but present in the crypts because of expression of redundant K7.[33,95] Omary and coworkers demonstrated that colitis in the K8-null model displays increased Th2 cytokines (IL-4, IL-5 and IL-13), as well as infiltration of CD4 positive cells in the submucosa. The phenotype amounts to a chronic Th2 colitis induced by a defect in the epithelium and not in immune cells, which lack keratins.[96] It is still unclear how intestinal epithelia recruit and activate CD4 positive cells. In these animals, no changes in paracellular permeability were detected in the distal large intestine by Omary and coworkers.[97] However, increased 3000 Da Dextran permeability was found in the small intestine,[52] suggesting a possible dependence of barrier disruption on the level of the gut. Unfortunately, detailed studies of intestinal permeability at various levels are missing. However, intestinal cells that express K8 or 18 bearing mutations identified in patients with Inflammatory Bowel Disease (IBD), showed an impaired barrier function, suggesting an epithelial cell-autonomous mechanism by which TJ permeability is dependent on IF.[98] Therefore, increased barrier permeability is a possible mechanism linking deficient keratin expression (or disease associated keratin mutations) and inflammation. Recent evidence from the skin seems to further support this possibility. Genes for K16 or 6, responsible for pachyonychia congenita, appear to display a close coregulation with genes that participate in the regulation of barrier function and innate immunity.[99]

In terms of transcriptional mechanisms, the K17 null mouse skin shows increased NF-kB activation in response to TNFα. While this can be interpreted as another example of keratin-dependent hyper-sensitivity to TNFα, it shows an indirect control of innate immunity response by specific keratins. K10 expression in the basal layer of the skin is another example of inhibition of NF-kB, possibly through inhibition of IKKβ and IKKγ expression.[100] We have recently shown that expression of constitutively active aPKC *inhibits* NF-kB activity in an epithelial cell line while *activating* it in a mesenchymal cell line.[101] Furthermore, aPKC is downregulated in IBD colon epithelia.[102] Bearing in mind that K8 null mice postranslationally downregulate aPKC,[52] it is possible that keratin-associated Hsp70 chaperoning also indirectly controls innate immunity activity in epithelia through aPKC. Conversely, it is well-known that inflammation increases keratin expression in pancreas, intestine,[95,103,104] and even ectopically, in cells which normally do not express keratins.[105]

Changes in cytokine expression are also associated with loss of keratin expression or disease-associated keratin mutations.

In skin tumor cells, CXC gene expression levels are controlled by K17 expression.[106]

In normal keratinocytes, K1 deficiency renders the cells mechanically weaker, but also increases expression of IL-18.[107] It is important to note that changes in the transcriptome occur in keratinocytes upon keratin deficiency. More importantly, these changes are specific for different keratins. K1 deficiency mimics the gene expression signature of atopic eczema and psoriasis.[107] K5 deficiency displays a different transcriptome signature,[107] which results in IL-6 and IL-1β expression.[108] Finally, the R156H K10 mutation, causal of a severe form of epidermolytic hyperkeratosis, results in increased expression of TNFα and RANTES, and reduced expression of IL-1β.[109]

While the precise mechanistic relationship between keratin expression (or mutations) and inflammation is still missing, these examples suggest that signaling mechanisms must be involved, and that various keratins may exert their anti-inflammatory effects at the transcriptional level via different pathways. In all known cases, however, there is a common pattern of anti-inflammatory activity of keratins. One may

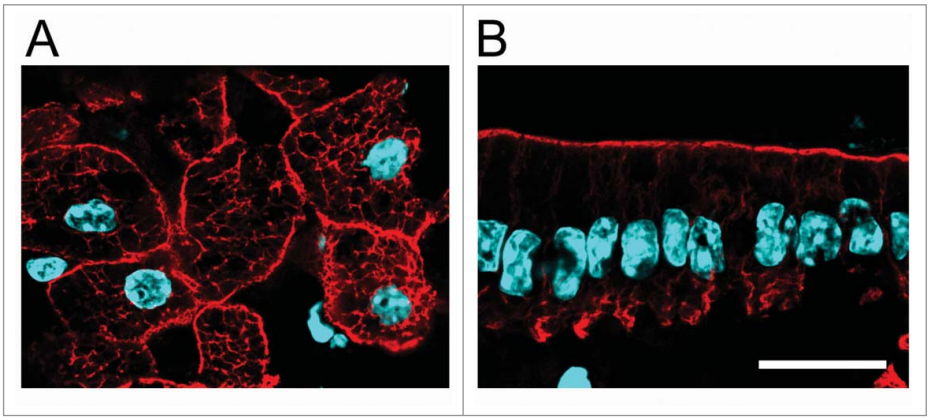

Figure 1. Polarity of IF in simple epithelia. A, B Frozen sections of formaldehyde-fixed liver (A) and small intestine epithelium (B) stained with anti-K8 antibody (red) and DAPI (light blue). Bar, 20 μm.

speculate that this is among the evolutionary advantages behind the great redundancy in keratin genes in vertebrates.

Keratins in epithelial polarity

The textbook image of keratin IF normally represents their subcellular distribution in keratinocytes (epidermis). In those cells the filaments fill the cytoplasm and extend from the nucleus to the plasma membrane. A similar non-polarized distribution is found in hepatocytes (Fig. 1A). In most other single-layered ("simple") epithelia and in epithelial cell lines such as MDCK and Caco-2, however, keratin IF are highly concentrated under the apical domain, showing thin extensions to the lateral domain to connect with desmosomes (reviewed in[110]). A small basal patch of keratins connects to hemidesmosomes[111,112] (Fig. 1B). The mechanism responsible for the subapical concentration of keratin IF remains elusive.

The question is whether the asymmetric distribution of keratins plays any role in apico-basal epithelial polarity. Published evidence shows that the formation and early polarization of epithelia in the embryo are not affected by lack of keratins.[88,113] This suggests that IF are not involved in the early acquisition of apico-basal polarity. Mistargeting of several polarized membrane proteins, however, was observed in K8 null intestinal epithelia.[33,97].Those results indicate the need of keratins for the maintenance of a normal polarized phenotype. Bearing in mind multiple scaffolding functions of keratin IF and the effects on signaling pathways described in the previous sections, understanding the molecular mechanisms that underlie the polarity defects is a challenge.

In principle, any of the scaffolding functions of keratin IF described before are expected to be asymmetrically concentrated under the apical domain, and, to some extent at the basal pole (Fig. 1B). As an example, scaffolding of Hsp70 chaperones is strictly dependent on K8 (and IF) expression in villus enterocytes. While there is a diffuse cytoplasmic Hsp70 signal, the chaperone becomes highly concentrated under the apical membrane (Fig. 2, arrow) (additional data in [52]). Likewise, it can be speculated that scaffolding of membrane molecules, perhaps through their cytoplasmic domains may control their traffic to the apical domain. Apically-bound membrane traffic vesicles need to cross through the dense layer of apical IF. Annexin II is essential for apical membrane traffic[114] and has been found to interact with keratin IF in the context of lipid rafts.[115] Another keratin-binding protein is Albatross, which in turn also binds Par3, a component of the polarity complex. Loss of keratins delocalizes Albatross and permits the invasion of basolateral proteins to the apical domain.[116]

A critically important apical protein, the cystic fibrosis conductance regulator (CFTR) was found to bind K18. Furthermore, the surface expression of CFTR is diminished in K18 null mouse gallbladder and duodenum[117] and in K8 null intestinal epithelia.[33] This suggests that membrane traffic can be positively regulated by keratins. Conversely, the glucose transporters GLUT1 and −3 are mislocalized away from the apical domain of keratin-null

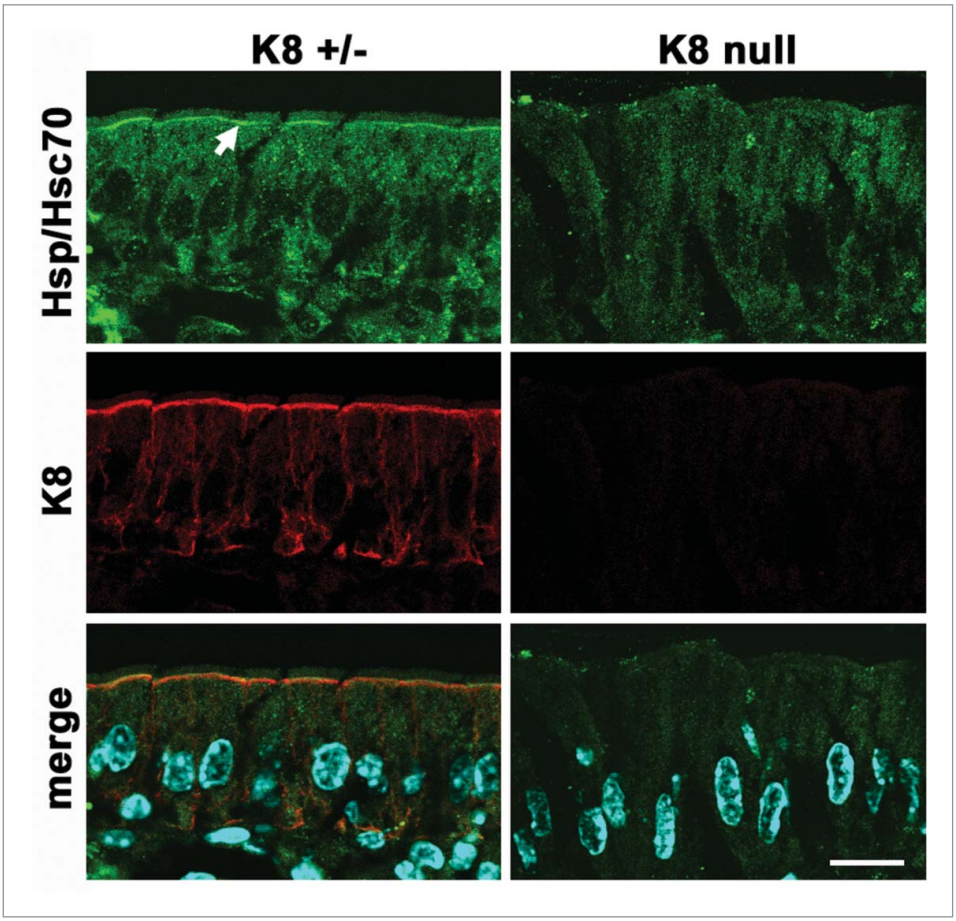

Figure 2. Polarized scaffolding of Hsp/Hsc70 in simple epithelia. Frozen sections of villus enterocytes from K8-null or heterozygous littermates were immunostained for K8 (red channel) or Hsp/Hsc70 (green channel). The arrow points at the apical concentration of the chaperone which is strictly dependent on the expression of keratin IF. Modified from.[52] Bar, 20 μm.

embryonic epithelia,[113] which deprives the cells of energy and activates AMPK, thus decreasing protein synthesis through mTOR inhibition. It is of note that the LKB1/AMPK pathway has been shown to control bile canaliculus (apical) formation in hepatocytes [118] as well. As mentioned before, another signaling mechanism, atypical PKC, is downregulated in K8 null epithelia, thus providing a possible explanation for both increased permeability of TJ and protein mistargeting. Furthermore, aPKC is also known to control surface localization of various glucose transporters in non-epithelial cells, including GLUT1.[119,120] While this function has not been demonstrated in epithelial cells, it remains a possible explanation for the results of Magin and coworkers in embryonic epithelia.[113]

Additionally, somewhat indirect mechanisms may also explain changes in apical polarity in keratin null phenotypes. Expression of plastin 1 (fimbrin), a keratin-binding protein, is necessary to maintain the structure of the apical terminal web, which comprises the highly-concentrated apical keratin IF.[121]

The distribution of apical microtubules is severely affected by the K8-null mutation, possibly through mislocalization of gamma-tubulin ring complexes.[122] In addition, activation of pro-inflammatory signaling may also play a role. In fact, apical mistargeting in K8-null colonocytes is partially reverted by treatment with antibiotics, which decreases the inflammatory response.[96] Although no data is currently available in the intestinal epithelium regarding innate immunity signaling in K8 null mice, in breast cancer cells in 3D cultures, inhibition of NF-kB by small molecules or shRNA induces apicobasal polarization.[123] Finally, the role of inflammation in the integrity of TJ has been reviewed elsewhere.[124] At least one polarity protein, Scribble, is delocalized under pro-inflammatory signals.[125] Accordingly, a relationship between innate immunity pathways and epithelial apicobasal polarity in the context of keratin deficient cells is worth further studies.

Challenges ahead to elucidate mechanistic aspects of IF function

The major challenge of IF research is that multiple functions, mechanical and non-mechanical, are affected simultaneously. Establishing a simple linear cause-effect relationship is, therefore, very difficult. The network of functions regulated by IF, in addition, may vary in different cell types. Loss of keratin expression or expression of keratin mutants associated with human disease results in increased sensitivity to apoptosis (in liver, placenta, and skin), changes in key signaling pathways, pro-inflammatory phenotype and partial loss of apico-basal polarity (intestine). Questions that remain unanswered, such as the examples that follow, are related to molecular mechanisms, cross-talk among them, and tissue specificity. How do IF protect epithelial cells from chemical stress, diminish innate immunity responses, and favor appropriate segregation of apical membrane proteins? The gaps of knowledge in this field are precisely at the interface between keratin molecules and interacting proteins involved in a substantial number of molecular mechanisms perturbed by loss of keratin function. Why do IF protect hepatocytes but not villus enterocytes from apoptosis? In both cases the loss of IF in the K8 null model is complete. There is no question that keratin IF contribute to the mechanical "stiffness" of epithelial cells,[90] but it is difficult to conceive that a hepatocyte may have harsher mechanical stresses than a small intestine villus enterocyte. That is especially true considering the fiber-rich diet of a rodent. Conversely, in the skin, at least one mutation (E477D K5p) does not impair mechanical characteristics of keratinocytes and yet causes EBS.[25]

The most obvious alternative to effects of cellular mechanical weakness is the scaffolding of several molecules which control intracellular signaling. From an evolutionary standpoint, this option makes sense to explain the formidable redundancy in type I and type II keratin genes developed during the evolution of chordates. Only one type I and one type II keratin gene would suffice to assemble filaments, as, for example, in early chordates.[126] However, many different keratin head and tail domains would be necessary to accommodate tissue-specific scaffolding in vertebrates.

Binding to keratin IF, however, is not an all or none phenomenon. Quantitative evaluation of scaffolding, (i.e., how much of the protein is bound to filaments and how much is free) is still needed in many cases. Likewise, quantification of the effects of scaffolding (i.e. how much protein bound to filaments is necessary to result in a certain change in a cell function) will be necessary to determine the impact of immobilization of cellular components on IF. This is especially important because keratin loss of function seems to affect multiple mechanisms. Accordingly it will be necessary to assess the relative importance of each one. While the current trend is to analyze effects at the cellular or animal level, there is a need for subcellular quantitative analysis as well, for example, separating keratin IF from the cytosol to measure function of the same molecules on the filament surface as opposed to the non-filamentous environment.[127] Ultimately, the interaction domains in keratins and each keratin binding protein will need to be determined. Molecular analysis at that level will confirm the conclusions from knockout models. In addition, it is conceivable that some interactions may be indirect. We can speculate that there is potential therapeutic significance in understanding mechanistically the interactions between keratins and signaling molecules. These interactions are expected to be specific to epithelia. In the same line of thought, because carcinomas still express keratins, it is likely that some of the keratin functions may still constitute a therapeutic target in cancer. For example, knockout of K14 in breast cancer cells abrogates invasiveness.[128] Understanding how K14 controls a differentiation program may help prevent metastasis. The implications for human health of understanding how keratins protect epithelia, therefore, may go beyond the numerous diseases caused or predisposed by keratin mutations.[129,130]

Abbreviations

Akt	Protein kinase B
aPKC	Atypical protein kinase C (isoforms ι/λ and νδ ζ)
Bag	BCL2-associated athanogene
DAPI	4′,6-diamidino-2-phenylindole
DEDD	Death Effector Domain Containing protein
EBS	Epidermolysis bullosa simplex
ERK	Extracellular Signal-regulated Kinase
Hsp	Heat shock protein
IBD	Inflammatory bowel disease
IF	intermediate filament
IKK	inhibitor of kappa B kinase

IL	Interleukin
K	keratin protein
Krt	keratin gene
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
NF-Kb	Nuclear factor kappa β
Par	PARtition deficient mutations in *C. elegans*. Homolog proteins in vertebrates
PKC	Protein kinase C
Rack	receptor for activated C-kinase
ROCK	Rho-associated protein kinase
TJ	tight junction
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain protein
ZO-1	Zonula occludens 1 protein, a tight junction structural component

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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