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Review

Multifaceted role of keratins in epithelial cell differentiation and transformation

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Keratins, the epithelial-predominant members of the intermediate filament superfamily, are expressed in a pairwise, tissue-specific and differentiation-dependent manner. There are 28 type I and 26 type II keratins, which share a common structure comprising a central coiled coil α-helical rod domain flanked by two nonhelical head and tail domains. These domains harbor sites for major posttranslational modifications like phosphorylation and glycosylation, which govern keratin function and dynamics. Apart from providing structural support, keratins regulate various signaling machinery involved in cell growth, motility, apoptosis etc. However, tissue-specific functions of keratins in relation to cell proliferation and differentiation are still emerging. Altered keratin expression pattern during and after malignant transformation is reported to modulate different signaling pathways involved in tumor progression in a context-dependent fashion. The current review focuses on the literature related to the role of keratins in the regulation of cell proliferation, differentiation and transformation in different types of epithelia.

Keywords. Cell proliferation; differentiation; keratins; posttranslational modification; squamous cell carcinoma; transformation

1. Introduction

1.1 *Keratins*

Keratins are epithelia predominant intermediate filament (IF) proteins that are expressed in a differentiation-dependent, tissue-specific and paired manner (Coulombe and Omary 2002). IF in epithelial cells are made up of keratin proteins that account for about 80% of the total protein content of stratified epithelia (Pekny and Lane 2007). They are characterized by unique physicochemical properties such as resistance to enzymatic digestion and are insoluble in dilute acids, alkali, water and organic solvents (Block 1951; Steinert *et al.* 1982). However, these proteins are soluble in solutions containing denaturing agents like urea or detergents like sodium-dodecyl-sulfate (Steinert *et al.* 1982). Keratins belong to a multigene family of proteins (Tomlinson *et al.* 2004). The first keratin protein nomenclature was published as a comprehensive keratin catalog by Moll *et al.* (1982). This classification was based on the profiling of keratins from normal human epithelial tissues, cell cultures and tumors using one- and two-dimensional gel electrophoresis (O'Farrell *et al.* 1977). This catalog included 19 members that were further classified into type I and type II keratins. Keratins with numbers 9 to 19 were classified as type I IF proteins. These are acidic (PI 4.5–5.5) with low molecular weight (40–56.6 kDa). Keratins with numbers 1–8 were classified as type II IF proteins. These are basic to neutral (PI 5.5–7.8) and have higher molecular weights (53–68 kDa). Additional keratins were subsequently identified in humans as well as in other species and the keratin catalog was updated a number of times (Moll *et al.* 1990; Takahashi *et al.* 1995). Differences in the molecular weight and PI of orthologous keratin protein in various species are attributed to the slight differences in the keratin genes, posttranscriptional modifications/posttranslational modifications (PTMs) in the processing of mRNA/proteins, or variations in the number of phosphorylated or glycosylated amino acid residues (Eckert 1988). After the completion of the human genome sequence, an updated nomenclature for

mammalian keratin genes and proteins is now available (Schweizer *et al.* 2006). It includes 28 type I (20 epithelial and 8 hair) keratins and 26 type II (20 epithelial and 6 hair) keratins.

1.2 *The structure of keratin proteins*

The keratin protein structure is characterized by a chain of amino acids and may vary in the number and sequence of amino acids as well as in polarity, charge, and size (Brown 1950; Makar *et al.* 2007). However, the amino acid sequence of a particular keratin is remarkably similar in different species (Makar *et al.* 2007). All keratins are composed of a central α-helical rod domain that is about 310–315 residues long and N- and C-terminal domains of variable size and chemical characters (Steinert *et al.* 1985) (figure 1). The variation in molecular sizes, isoelectric points, and immunogenicity of keratins is almost entirely due to the different sizes of the end domains.

1.3 *Interaction of keratins with membrane proteins*

Desmosomes are cell–cell anchoring junctions, whereas hemidesmosomes (HDs) are cell–extracellular matrix (ECM) junctions that connect the basal surface of epithelial cells to the underlying basal lamina. Intracellularly, armadillo family proteins such as plakoglobin and plakophilins and plakin proteins such as desmoplakin anchor to IFs at a desmosomal site, whereas at the HD front, hemidesmosomal linker proteins, BPAG1e and plectin, anchor epithelial keratins (e.g. K5/14) to the cell surface via α6β4 integrin (figure 2) (Jones *et al.* 1998; Desai *et al.* 2009; Chaudhari and Vaidya 2015).

1.4 *PTMs of keratins in physiology and pathology*

Like other IFs, the functions of keratins are also orchestrated by various PTMs, including phosphorylation, glycosylation, SUMOylation etc (Omary *et al.* 1998). Six major PTMs of keratins are detailed below.

1.4.1 *Glycosylation:* The addition of a single *N*-acetyl glucosamine (GlcNAc) to serine and threonine residues of nuclear and cytoplasmic proteins is termed as OGlcNAcylation. Two enzymes, O-GlcNAc Transferase and O-GlcNAcase, are known to regulate this dynamic process. Similar to other cytoplasmic and nuclear proteins, various keratins have been identified to undergo glycosylation, such as K13, K8 and K18 (Chou *et al.* 1992; Ku and Omary 1995). The major role of keratin glycosylation in simple-type epithelia is to facilitate the phosphorylation and activation of cell survival kinases during stress and injury (Ku *et al.* 2010). Moreover, O-GlcNAcylation has been postulated to be a nutrient sensor and consequently has an important role in signal transduction (Ku *et al.* 2010; Rotty *et al.* 2010).

1.4.2 *Transglutamination/transamidation:* Transglutaminase-2 is an inducible acyltransferase that catalyzes the formation of the amide bonds (transamidation) between the ε-amino group of lysine and the γ-carboxyl group of glutamine (Nemes *et al.* 2005). Transamidation seems to be essential for the attachment of several epidermal type II keratins to the cornified envelope of the skin, which performs a critical barrier function (Candi *et al.* 1998). In the physiological context, the role of this modification is clear in terms of providing a compact protective structure (Omary *et al.* 1998). This modification is identified in both epidermal as well as simple epithelial keratins.

1.4.3 *Sumoylation:* SUMOylation is a modification analogous to ubiquitylation and involves the addition of small ubiquitin-like modifiers. Lysine residues on various proteins including nuclear and cytoplasmic IFs (keratins) are known to undergo SUMO (SUMO1, SUMO2 and SUMO3) conjugation in a covalent and reversible manner (Alonso *et al.* 2015). Keratin SUMOylation like other IFs potentially regulates their filament formation and solubility. Disease causing alteration in SUMOylation affects these properties of keratins (Snider *et al.* 2011).

1.4.4 *Ubiquitylation:* The ubiquitin–proteasome pathway (UPP) is involved in regulating the cell cycle, signal transduction, differentiation, and stress response. The ubiquitin ligases CHIP/STUB1 are shown to target mutant keratins for

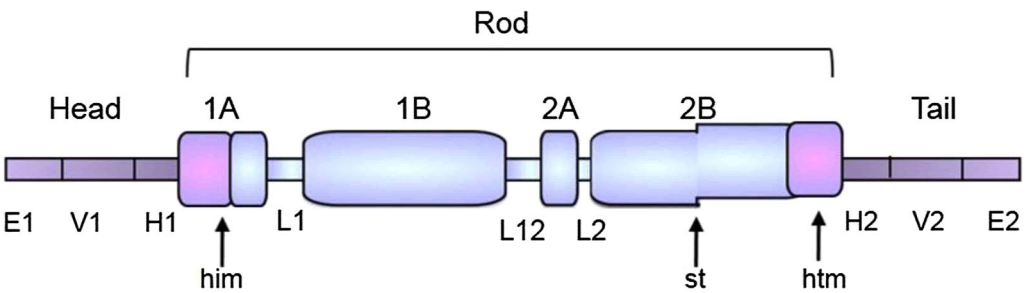

Figure 1. A schematic picture showing the secondary structure of keratins.

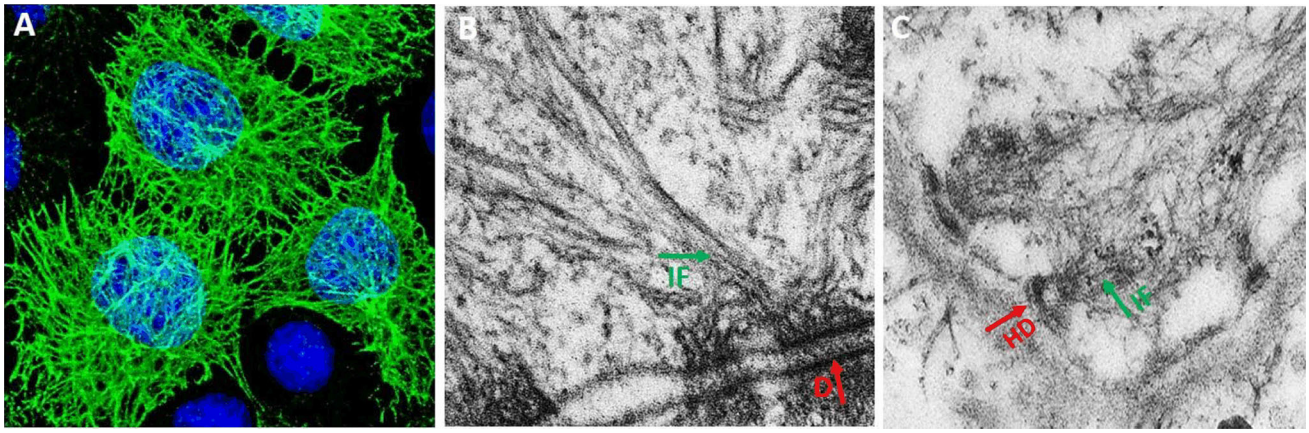

Figure 2. Interaction of keratins with membrane proteins in oral epithelial cells. (**A**) Immunofluorescence-stained image of human oral buccal mucosal epithelial cultures showing the organization of the IF network in the cytoplasm that connects to the cell–cell contacts at the plasma membrane. Green: IF, Blue: nucleus. Magnification 400×. (**B**) Transmission electron micrograph of human oral buccal mucosal tissue section showing cytoplasmic IF (keratins) connecting to the desmosomes at the cell–cell adhesion junctions. D: Desmosome. Magnification 20,000×. (**C**) Electron micrograph of human oral buccal mucosal tissue section showing cytoplasmic IF (keratin) interacting with hemidesmosomes at the cell–ECM adhesion junctions. HD: Hemidesmosome. Magnification: 10,000×.

degradation (Loffek *et al.* 2010). The ubiquitination sites on keratins are not clearly identified but there are some putative lysines that are identified via mass spectrometry (Kim *et al.* 2011). Keratin, the predominant IF expressed in epithelial cells, is highly dynamic and responds to injury, sometimes in the form of degradation of the keratin IF network. According to an earlier report, in A549 cells, shear stress results in the disassembly and degradation of keratin proteins via the UPP (Jaitovich *et al.* 2008). Nonetheless, the obligatory heterodimeric nature of keratins also suggests that one of the keratin monomers might be degraded by the UPP in the absence of its partner, which usually performs a stabilizing function. The accumulation of ubiquitylated IF proteins occurs in the context of cellular dysfunction, which is accompanied by proteasome inhibition (Rogel *et al.* 2010).

1.4.5 *Acetylation:* Protein lysine acetylation is a reversible process involving the modification of ε-amino groups of lysine residues with an acetyl moiety from acetyl-CoA. The dynamics of this process are regulated by specific enzymes carrying out lysine acetylation and deacetylation in response to different stimuli (Verdin and Ott 2015). According to a previous study based on proteomic data and site-directed mutagenesis, Lys-207 has been identified as a major acetylation site on human K8 in the rod-domain. Keratin IFs were demonstrated to be dynamically regulated by lysine acetylation and SUMOylation in response to cellular energy status and tissue injury. Keratin acetylation provides a new mechanism to regulate keratin filaments, possibly via modulating keratin phosphorylation (Snider *et al.* 2013).

1.4.6 *Phosphorylation:* Amongst all the PTMs, phosphorylation is one of the best-studied, highly dynamic and multifunctional keratin modifications (Snider and Omary 2014). Mostly, serine is the primary amino acid of keratins that undergoes phosphorylation. The serine phosphorylation sites are targets for several protein kinases, including members of the MAP kinases such as p38, ERK, PKC, cAMP, JNK and phosphatases like PRL-3 and PP2A. These kinases and phosphatases together regulate keratin functions under specific physiological conditions (Toivola *et al.* 1997; He *et al.* 2002; Omary *et al.* 2006; Tao *et al.* 2006). Several serine/threonine phosphorylation sites and some of the relevant kinases have been characterized in case of K1, K8, and K18. Keratin phosphorylation associated functions include filament organization/reorganization, protection against cell stress, cell signaling, apoptosis, and cell compartment-specific roles. The role of keratin phosphorylation in cell transformation/progression has been discussed at the end of this review.

2. Keratin expression is differentiation dependent and developmentally regulated

Keratin expression starts early during embryonic development (Franke *et al.* 1982). In mice, keratin proteins have been first detected at the 4-cell stage and proper filament assembly was observed at the 16-cell stage (Chisholm and Houliston 1987). At a certain stage of embryonic development, fetal keratin expression changes and site-specific adult expression begins. Keratin expression in human epithelial cells has been studied during development in different epithelia such as the epidermis, lung, trachea, breast, stomach, intestinal epithelia, etc. Their expression is regulated in a tissue-specific and differentiation-dependent manner. The cells of simple epithelia express K8/18 while all the stratified squamous epithelia express keratins 5 and 14. In the stratified epithelia, keratins express a different pattern, tightly regulated by the differentiation program of the tissue. K5

and K14 are expressed in the basal layer of the stratified epithelia (Moll *et al.* 1982). As these basal cells differentiate, the K5/14 expression is gradually reduced and is taken over by the expression of new pair of keratins depending upon the tissue type (Fuchs and Green 1980). Differentiating cells express K1/10 in the skin, K3/12 in corneal cells and K4/13 in internal stratified epithelia (figure 3) (Albers 1996).

Keratin 8/18 is the first pair to be expressed during embryogenesis and later its expression is restricted to simple epithelia and is predominantly seen in the epithelial components of the glandular tissues, including the pancreas and the intestine (Ku *et al.* 1999). In the pancreas and the intestine, this pair is often seen with other keratins such as K7, K19, K20. Furthermore, K8/18 is also seen in the mixed epithelia such as breast and lung. In the breast epithelia, the basal/myoepithelium cells express K5/14. These basal myoepithelium cells represent the proliferating compartment. On the other hand, in the differentiation compartment, luminal cells express K8/18 (figure 3) (Moll *et al.* 1982; Buhler and Schaller 2005).

3. Functions of keratins

The primary function of keratins is to impart mechanical strength to cells and maintain the cell shape and tissue integrity (Kirfel *et al.* 2003). Furthermore, genetic and molecular analyses revealed that point mutations in highly conserved amino or carboxyl terminal ends of the rod domains of keratin led to autoimmune skin blistering diseases like epidermolysis bullosa simplex (mutations in keratin 5/14 genes), epidermolytic hyperkeratosis (mutations in keratin 1/10 genes), and epidermolytic palmoplantar keratoderma (mutations in the keratin 9 gene) (Vaidya and Kanojia 2007). The effects of a mutation in a particular keratin are more deleterious due to protein aggregation rather than the absence of that keratin probably because it would also result in defective interactions with membrane proteins, affecting their function of imparting structural resilience. Similar effects have been observed as a result of mutations in keratin-associated proteins such as plectin and desmoplakin (Wiche 1998; Omary *et al.* 2004; Liu *et al.* 2012). Well-established structural functions alone cannot explain the diversity and dynamic nature of keratin filaments. Several questions pertaining to the multiplicity of keratins and their probable tissue function remain unanswered. These unresolved issues include:

(a) If keratins have only a structural function, what is the need for such a wide range of keratins?
(b) Why do they exhibit tissue-specific expression?
(c) What are the factors regulating their tissue-specific expression?
(d) Do keratins have regulatory roles?

The studies conducted over the past two decades to understand the multiple functions of keratins have indicated that they modulate processes such as osmolarity and apoptosis and regulate protein synthesis (Gilbert *et al.* 2004; Toivola *et al.* 2005; Kim and Coulombe 2007). In addition, various experimental evidence in recent years has revealed many more complex functions of keratins, such as intracellular organelle transport, intracellular communication, cell–cell contact, translation control, proliferation, differentiation, various stress responses, cell signaling and malignant transformation (Magin *et al.* 2007). This diversity of epithelial functions may answer why distinct keratins genes are evolved. These studies have also answered some of the questions that have been raised above. In the present review, our focus is on the function of keratins in cell differentiation, proliferation and transformation/progression. All of these processes are regulated by highly complex patterns of

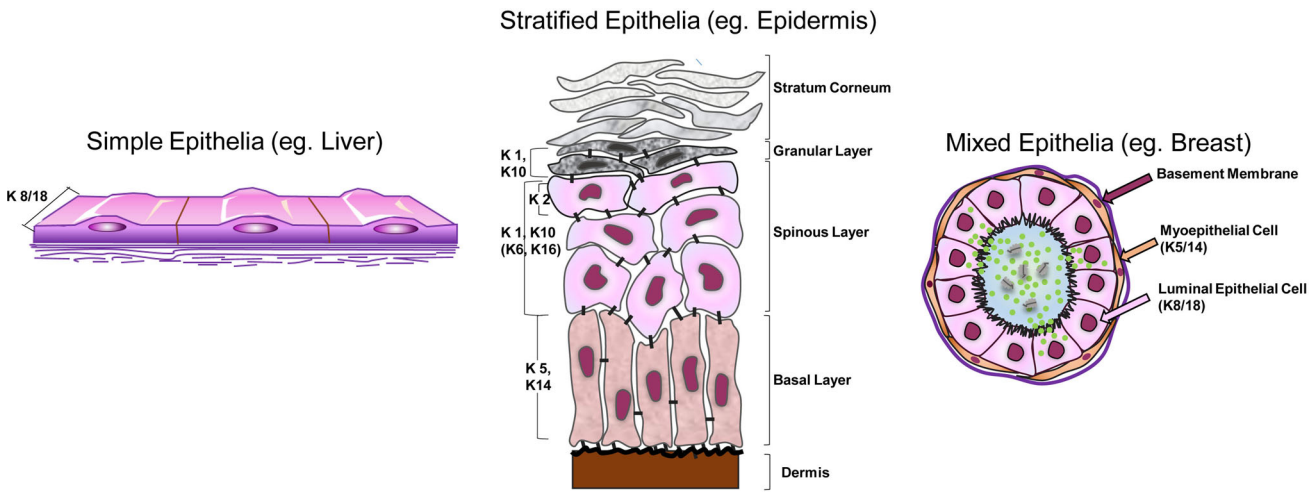

Figure 3. Keratin expression is tissue type and differentiation dependent (adapted and modified from Porter and Lane 2003; Adriance *et al.* 2005).

phosphorylation and molecular associations of different keratin-associated proteins (Magin *et al.* 2007).

4. Keratins in cell proliferation

4.1 *Role of K5/14 in epidermal cell proliferation*

K5/14 are expressed in the basal layer of the epidermis, which comprises epidermal stem cells and transient amplifying cells (Coulombe *et al.* 2004). Alam *et al.* demonstrated a significant reduction in proliferation in HaCaT and in an oral squamous cell carcinoma (OSCC)-derived cell line AW13516 cells in response to downregulation of the K5/14 pair (Alam *et al.* 2011c). They attributed the reduction in cell proliferation to delay in cell cycle progression. In addition, there was a reduction in the S-phase marker Ki67 and a reduction in the levels of cyclin D1 (G1–S phase-specific cyclin) and proliferating cell nuclear antigen (S-phase specific marker). Increase in p21 and p27 was also observed in K14 knockdown cells. Furthermore, reduction in the K14 knockdown cells entering the M-phase was observed. The delayed cell cycle progression was correlated with a decrease in phosphorylation of Akt at serine473 in K14 knockdown cells. These observations together suggest that K5/14 play an important role in regulating cell proliferation in the basal cells of the stratified epidermis via the PI3K/Akt pathway (Alam *et al.* 2011c).

4.2 *Role of K6/16 in cell proliferation*

K6/16 is constitutively expressed at low levels in a number of stratified epithelial cells such as palmar and plantar epidermis, tongue, oral mucosa and the outer sheath of hair follicles. The directed expression of K16 using the K14 gene promoter in the progenitor cells of transgenic mice displays a dramatic postnatal phenotype that is characterized by skin that is hyperkeratotic, scaly and devoid of fur. The phenotype was normalized 5 weeks after birth. The hyperproliferative phenotype was attributed to the increase in the phosphorylation of EGFR (Paladini and Coulombe 1998). The authors further concluded that the expression of K16 leads to changes in cell signaling of keratinocytes which were attributed to the C-terminal tail domain since there were no changes in the control mice expressing the K16–K14 chimeric protein. In addition, the K6/16 expression is also seen in stratified epithelial cells featuring hyperproliferation, such as psoriasis (McGowan and Coulombe 1998a). Psoriasis is a chronic skin inflammatory disease characterized by keratinocyte hyperproliferation of epidermis. A recent study has shown that Nrf-2 transcription factor regulates the expression of K6, K16 and K17 in psoriasis. Nrf-2 promoted the expression of K6, K16 and K17 by binding to the ARE domain located in the promoter of these genes. In mice with imiquimod-induced psoriasis-like dermatitis, topical application of Nrf-2 small-interfering RNA alleviated the epidermal hyperplasia with reduced expression of these keratins, suggesting that Nrf-2 is responsible for an increase in the expression of these keratins (Yang *et al.* 2017).

4.3 *K17 regulates cell growth through the Akt/mTOR pathway*

The role of K17 in epithelial cell growth has been depicted via the Akt-mTOR pathway using knockout mouse model (Kim *et al.* 2006). Keratin 17 is rapidly induced in wounded stratified epithelia. K17 null keratinocytes obtained from K17 null mice demonstrated a decrease in amino acid incorporation into newly synthesized protein and a delay in peptide elongation. Akt and mTOR activity showed a significant decrease in K17 null keratinocytes as compared with WT cells. K17 regulates cell growth by binding to the adapter protein 14-3-3σ. The 14-3-3 proteins belong to a seven-member family of highly conserved adapter proteins that modulate the subcellular distribution and activity of >100 proteins, mostly in a serine/threonine phosphorylation-dependent manner (Hermeking and Benzinger 2006). Kim *et al.* demonstrated that 14-3-3σ colocalizes with K17 in the cytoplasm of the keratinocytes and their association is phosphorylation dependent (Kim *et al.* 2006). Two amino acid residues located in the amino terminal head domain of K17 are required for the relocalization of 14-3-3σ from the nucleus to the cytoplasm and for the concomitant stimulation of mTOR activity and cell growth. Thus, K17 could regulate keratinocyte growth during skin development and homeostasis.

Kb6a, Kb6b and K17 were found to be upregulated in wound proximal epidermal keratinocytes in addition to being normally expressed in epithelial appendages (McGowan and Coulombe 1998b; Paladini and Coulombe 1998; Kim *et al.* 2006). K17 knockout embryos showed delayed wound closure while K6a and K6b double knockout embryos failed to show any difference in the wound healing potential. In wild-type embryos, epithelial cells close to the wound upregulate the expression of K17 and become significantly large in size. This hypertrophic response is markedly reduced in $K17^{-/-}$ mice but not in $K6a^{-/-}$ and $K6b^{-/-}$ double knockout embryos. This was due to the defect in the translation, correlating with a decrease in the Akt-mTOR signaling, as discussed above (Kim *et al.* 2006). In contrast, Kb6a and Kb6b genetic ablation results in enhanced keratinocyte migration. Rotty *et al.* attributed this phenotype to the activation of Src kinase. Src was bound to the K6 filament through its SH2 domain in a phosphorylation-independent manner, resulting in kinase inhibition. Thus, K6 negatively regulates Src kinase activity and regulates migration during wound repair (Rotty and Coulombe 2012).

4.4 *Role of K10 in cell proliferation*

Another study using the ectopic expression of K10 demonstrated that there is inhibition of cell proliferation in human

keratinocytes in culture, whereas K16 appears to promote the proliferation of these cells. Cell cycle analysis of these cells shows that K10 expression leads to an increase in the G1-phase population and a decrease in the S-phase population. Conversely, K16 exhibited an increase in the S-phase population and reduced fraction of cells in the G1-phase. K10 inhibition is reversed by the cotransfection of K16 but not that of K14. These expression patterns are coherent with the actual expression patterns of these proteins in the stratified epithelia. The basal proliferative keratinocytes express K14 and when they terminally differentiate, they switch off K14 and start expressing K10.In contrast, in response to the hyperproliferative stimuli, K16 replaces K10. K10-induced inhibition of cell proliferation requires functional Rb proteins. K10 alone could not induce cell growth arrest in these cell lines, but when K10 was co-transfected with pRb or p107, the inhibition was restored. pRb phosphorylation and cyclin D1 expression are reduced in K10-transfected cells and are increased in K16-transfected cells. Using K10 deletion mutants, mapping of this inhibitory function to the nonhelical terminal domain of K10 demonstrated that the presence of one of these domains is sufficient to promote cell growth. Another study using K10-transfected keratinocytes and forced transfection in the basal cells of transgenic mice has shown that K10 inhibits cell cycle entry by forced sequestration of Akt and protein kinase C in an Rb-dependent manner (Paramio *et al.* 1999; Santos *et al.* 2002).Another study showed that K10 functions as a negative modulator of cell cycle involving the PI3K-signaling pathway, as shown by the transfection of PDK1, Akt, and PKCζ with K10, respectively. It rescued the inhibitory effect of K10. Furthermore, functional and biochemical studies demonstrated that the interaction between K10 and these kinases, i.e. PKCζ and PKB, involves the non-α-helical amino domain of K10 (Paramio *et al.* 2001). Santos *et al.* further extended the *in vitro* studies to the *in vivo* situation. Their work analyzed the alterations seen in transgenic mice that ectopically express K10 in the proliferative basal layer of the epidermis. Increased expression of K10 leads to the hypoplastic and hyperkeratotic phenotype due to drastic decrease in the proliferation of the keratinocytes. They also attributed the phenotype to the association of Akt and PKC to K10, thus inhibiting their activities. This demonstrates that the *in vivo* function of K10 includes control of epithelial proliferation in skin epidermis (Santos *et al.* 2002). On the other hand, Reichelt *et al.* have shown no epidermal Akt activation in K10 knockout mice. The authors have suggested that this may be due to the fact that Akt activation is restricted to the basal layer of the epidermis which is why it is not seen in the suprabasal layer (Reichelt and Magin 2002).

The evidence from the above studies of knockout and transgenic animals proves that keratin filaments are not only important for structural support but also play an important role in cell proliferation, wound repair, protein synthesis, and epithelial cell growth in a context-dependent manner.

5. Role of keratins in cell differentiation

5.1 *Role of K5/14 in regulating epidermal cell differentiation*

During epidermal cell differentiation, when cells move from the basal layer to the suprabasal layer, K5/14 is downregulated and the expression of differentiation markers such as K1/10 and involucrin is induced (Fuchs and Green 1980). Dakir *et al.* developed transgenic mice expressing human K14 in airway progenitor cells using the mouse Clara cell-specific 10 kDa protein (CC10) promoter. The authors demonstrated that CC10 hK14 induced squamous differentiation program in the lung epithelium but failed to promote squamous maturation, suggesting that K14 may have a role in squamous cell differentiation (Dakir *et al.* 2008). Lloyd *et al.* have shown that there were no alterations in terminal differentiation during fetal development in K14 and K5 knockout mice (Lloyd *et al.* 1995; Peters *et al.* 2001). It is possible that K14 may not be regulating differentiation in fetal development, but K5 and K14 may act as modulators of terminal differentiation in the adult stage.

Studies from our laboratory have shown that upon the downregulation of K14 in HaCaT (derived from human adult skin) and AW13516 cells (derived from human squamous cell carcinoma of tongue), there was an increase in the cell differentiation markers such as involucrin and K1. Notch-1, a key modulator of the squamous cell differentiation process, was also found to be elevated in K14 knockdown cells, both at the surface as well as at the nuclear level. An increase in the activated Notch-1, that is the Notch-1 intracellular domain (NICD), was also observed. These results suggest that K14 downregulation leads to an increase in NICD which further modulates the levels of differentiation markers such as involucrin and K1. Thus, it was concluded that K14 is a negative regulator of cell differentiation (Alam *et al.* 2011c). A recent work in our laboratory has shown that K14-regulated cell differentiation involves TAp63, which in turn modulates Notch-1 expression as well as NICD levels and hence differentiation (Srivastava *et al.* 2018).

5.2 *Role of K10 in epidermal differentiation*

In another study, when the K10 transgene was expressed in thymus tissues under the K5 promoter, there was a reduction in cell proliferation, an alteration in the differentiation pattern, and an increase in apoptosis. The molecular characterization of this phenotype demonstrated reduced Akt activity and reduced Notch-1 activity in thymus epithelial cells. This suggests that K10 has a role to play in cell differentiation (Paladini and Coulombe 1998; Santos *et al.* 2005). Targeted deletion of K10 in mice alters differentiation of sebocytes. At the molecular level, there was no change in the expression of β-catenin and its target cyclinD1 and

c-Myc. The authors concluded that the altered composition of the suprabasal IF in $K10^{-/-}$ increases the differentiation of epidermal stem cells towards sebocyte lineage (Reichelt *et al.* 2004a).

Previous studies have shown the role of K16 and K10 in modulating cell proliferation/differentiation in the transgenic mouse model (Paladini and Coulombe 1998; Santos *et al.* 2002; Koch and Roop 2004). In another study, chimeric protein, which consists of the K14 rod domain fused to the K10 head and tail domains (K1014chim), was expressed in the basal sheath of hair follicles. Interestingly, K10 end domain did not have any effect on basal cell proliferation *in vivo*. The mutant mice demonstrated increased susceptibility to benign tumor formation when subjected to the chemical carcinogenesis protocol. The authors further found that the increase in tumor burden was due to a decrease in resistance to apoptosis. Tamiji and colleagues also provided evidence that K10-expressing HaCaT cells appear to be partially protected from chemically induced apoptosis (Tamiji *et al.* 2005). Thus, the authors speculated that the function of K10 is to inhibit apoptosis for the timely differentiation of keratinocytes (Chen *et al.* 2006).

One more study using $K10^{-/-}$ mice has shown an increase in hyperproliferation of the basal cells and an increase in cell size. No cell fragility was observed. There was an increase in the expression of c-Myc and cyclin D1. The stimulation of basal cell proliferation after the loss of K10 must involve signaling from the suprabasal cell to the basal cell compartment of the epidermis. This could involve paracrine secreting cytokines such as KGF, GM-CSF, IL-1, and/or members of TGF-β superfamily (Reichelt *et al.* 2004b). Thus, K10 inhibits basal cell proliferation and induces differentiation of keratinocytes.

5.3 *Role of keratin 8 in differentiation*

K8/18 pair is expressed in cells of simple epithelial tissues and is not normally expressed in the stratified epithelia. It is also expressed in mixed epithelia such as lung and breast and is associated with cell differentiation. In skin, K8/18 is not detected in the epidermis. However, the cells of the hair follicle may sporadically express K8 and Merkel cells are K8/18 positive. When the human *K*8 gene was ectopically expressed in the basal layer of the epidermis, the resulting TGKH8 mice demonstrated hyperplasia and hair follicle dysplasia that progressively developed into premalignant areas in the aging animals. They also showed abnormal epidermal differentiation and a dramatic increase in the malignant progression of the skin tumors. Fillagrin, a marker of cell differentiation, was expressed in the granular cells of the epidermis in wild-type mice. However, in transgenic mice, the fillagrin was also seen in the dysplastic hair follicles. Staining for loricrin and involucrin, major precursors of the cornified epithelium, was increased in the dysplastic hair follicles of transgenic mice. Hyperplastic epithelium bordering also demonstrated an increase in the loricrin expression in transgenic mice. Furthermore, there was an aberrant expression of K6 in the dysplastic hair follicles, without any increase in their proliferative rate, suggesting that they follow an alternative differentiation pathway. Thus, the expression of K8 in skin abrogates the differentiation status of the epidermal and follicular cells (Casanova *et al.* 2004).

Thus, keratins play an important role in cell proliferation and differentiation. At the molecular level, the signaling molecules controlling the expression of keratins and their regulation are the areas still being explored.

6. Keratins in transformation

Traditionally, keratins have been used to identify the epithelial origin of a cell since even in epithelial pathologies/malignancies, the expression pattern of the native keratin pair is retained. Interestingly, along with the cell-type-specific keratin, malignant tumor tissues also aberrantly express keratins of other epithelial origins. Lately, the appearance of a cleaved or full-length keratin in circulation is also used for cancer prognostication. These aberrantly expressed keratins actively/passively contribute to conferring a transformed/malignant phenotype to the cell. The emerging evidence on aberrantly expressed keratins highlights their role in the diagnosis/prognosis of cancer, in the regulation of transformation and cancer progression (metastasis), and in therapeutic targeting and responsiveness to treatment (figure 4).

6.1 *Keratins as indicators of epithelial transformation*

Keratins being expressed in a cell type and differentiation stage-specific manner qualify to serve as potential markers to sense the abnormalities in the epithelia. Hence, their expression has a strong diagnostic and prognostic potential. Keratin typing/fingerprinting is widely used to identify simple and stratified epithelia, to characterize normal and transformed epithelia, to mark invasive and noninvasive tumor margins, and to predict survival of the cancer pateints. For example, in adenocarcinomas, which are carcinomas of the glandular epithelia, the expression of simple epithelial-specific keratins K8, K18 and K19 is normal, while the variable expression of K7 and K20 is aberrant (Matros *et al.* 2006; Bonora *et al.* 2015). Here, the expression of simple keratins is indicative of the origin of tissue while aberrantly expressed keratins are markers of the neoplastic phenotype. Conversely, both simple epithelial-specific keratins (K7, K8, K18 and K19) and stratified epithelial-specific keratins (K13 and K20) are expressed in transitional cell carcinomas (Moll *et al.* 1988). Bloor *et al.* have characterized the changes in the expression of differentiation-specific keratins from oral dysplasia to OSCC, both at the transcript and protein levels.

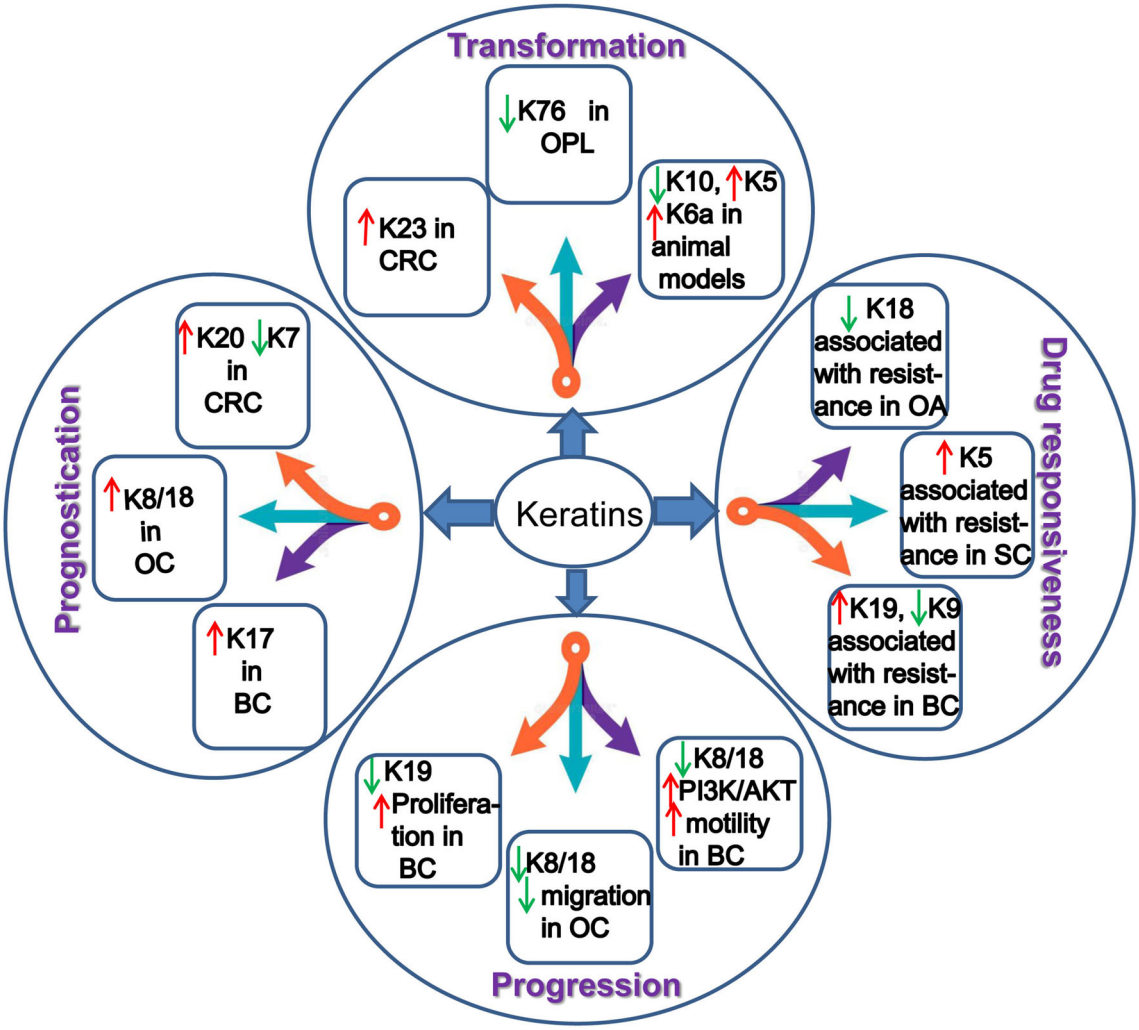

Figure 4. Schematic displaying the role of keratins in cancers with respect to prognostication, transformation, progression, and drug responsiveness. (Keys: OPL: oral premalignant lesions; CRC: colorectal cancers; BC, breast cancers; OA, ovarian adenocarcinomas; SC, serous ovarian cancers.).

They found the expression of both K1/K10 and K4/K13 pairs in mild dysplasia, while in moderate dysplasia, the expression of K1/K10 dominated the expression of K4/K13 (Bloor *et al.* 2000). Complete loss of these pairs of keratins was observed in severe dysplasia and in poorly differentiated squamous carcinoma, indicating the strong association of these keratins with epithelial cell differentiation (Bloor *et al.* 2001). On similar lines, loss of K13 and gain of K17 is shown to significantly correlate with oral epithelial malignancies (Mikami *et al.* 2011). Likewise, loss of K7 and overexpression of K20 is common in colorectal cancers (CRCs), while reduced expression of K20 correlated well with the poorly differentiated tumors of the same type (Harbaum *et al.* 2012). Also, in breast cancers, K17 expression alone is associated with poor prognosis (van de Rijn *et al.* 2002). Our study on oral potentially malignant lesions and OSCCs has shown loss of K5 and aberrant expression of K1, K8 and K18. The correlations with clinicopathological parameters showed that the expression of K1, K8 and K18 is associated with poor survival and higher risk of recurrence (Sawant *et al.* 2014). Furthermore, keratin fragments are also released in circulation as a result of cancer cell death and can be used as potential biomarkers to identify patients with systemic disease and/or with micrometastases. In patients with CRC, the presence of soluble fragment M65 in circulation was shown to be associated with malignancy (Ausch *et al.* 2009). Similarly, the presence of the K19 fragment (known as CK19-2G2) in the sera of preoperative and postoperative lung patients was shown to be a potential indicator of the responsiveness of patients towards the treatment (Gao *et al.* 2014). Expression of K34betaE12/K7 was shown to be a prognostic marker for resected early-stage non-small cell lung cancer. This expression status can be used to select high-risk patients with poor prognosis (Pohl *et al.* 2016). Our laboratory has also shown the prognostic significance of serum fragments of CK8, 18, and 19 (TPA

assay) in human oral cancer. The higher TPA levels even after surgery showed prognostic significance (Sawant *et al.* 2011).

Overall, keratins are emerging as sensors of the changing epithelia. Their characteristic expression pattern (which is tissue specific and differentiation specific) makes them bonafide markers of those particular epithelia. Furthermore, their presence or absence in a context-dependent fashion allows them to be used as markers for certifying epithelia as normal or abnormal. Increasing evidence has highlighted their potential to predict recurrence, micrometastasis, and survival. Therefore, it would be beneficial to use keratin typing as an adjunct to histodiagnosis to identify the origin and severity of the disease with a higher degree of accuracy.

6.2 *Keratins as regulators of transformation*

Growing evidence suggests that keratins may not merely be indicators that sense the changes in epithelia but may also be actively involved in driving the change. They play a regulatory role in maintaining cellular homeostasis (Vaidya and Kanojia 2007). Hence, downregulation of cell type specific keratin or aberrant expression of keratin in a given epithelia results in the modulation of various signalling pathways. This has prompted researchers to investigate their contribution in the development of tumor. In this direction, the expression of K76 (which is a resident keratin of suprabasal epithelial cells of the hard palate and gingiva) was seen to be downregulated in oral precancerous lesion, OSCC and in a sequential progression of hamster model of oral carcinogenesis. Furthermore, authors have suggested that K76 downregulation is associated with a hyperproliferative phenotype but not sufficient to result in transformation (Ambatipudi *et al.* 2013). In line with this notion, K10 downregulation is widely associated with human skin carcinomas and mouse carcinogenesis model (Roop *et al.* 1988). One of the mechanisms in which the absence of K10 may contribute to tumorigenesis would be through the inhibition of cell proliferation since K10 null mice showed epidermal hyperproliferation through the induction of c-Myc (Reichelt and Magin 2002). Conversely, the overexpression of K10 was able to inhibit cell proliferation through the sequestration of AKT (Paramio 1999). Very recently, K23, which is a type I acidic keratin was shown to play a role in the growth of human CRC. It is demonstrated that K23 upregulates the expression of human telomerase reverse transcriptase (hTERT) and that is how it helps in driving the growth of CRCs. Furthermore, tumors showing increased expression of both K23 and hTERT had shorter overall survival. This demonstrates a pro-oncogenic function of K23 in CRCs (Zhang *et al.* 2017). In Ewing sarcoma, K17 has also been shown to play a central role in two independent cancer-related phenotypes: cellular transformation and cellular adhesion. It is shown to play a coordinating function by inducing AKT signaling to mediate cellular adhesion and regulating transformation independent of AKT signaling (Sankar *et al.* 2013). Similarly, we have reported the malignant transformation of cells derived from human fetal buccal mucosa upon forced expression of K8. Forced expression of K8 in these cells resulted in the formation of K8/18 filaments, colonies in soft agar and subcutaneous tumors in nude mice as well as metastasis in lungs. On similar lines, we also found increased protein levels of K5/K6a and aberrant expression of K8 at various stages of the chemically induced rat lingual carcinogenesis, right from dysplasia to papilloma to carcinoma. These observations suggest that the expression of K8 may begin early in the development of oral cancer and the expression of K8 in potentially malignant lesions may be used to identify the high-risk lesions from the other lesions (Kanojia *et al.* 2012). Although the exact mechanism underlying the K8-mediated transformation is unclear, it appears that an aberrant expression of K8 may not be just a passive change in the process of oral oncogenesis.

6.3 *Keratins in cancer progression*

Extensive studies have reported the aberrant expression of keratins in several cancers and their association with poor survival. However, recent literature supports the notion that keratins are perhaps involved in regulating the progression of cancers by interacting with an array of molecules to ultimately govern the pathways driven by them. In recent years, the role of keratins in cancer cell adhesion, migration, and invasion along with their underlying mechanisms has been increasingly investigated. This will allow to specifically target keratins and their associated molecules to control/treat cancers. Keratins may play a tumor-suppressive or a tumor-promoting role, which is essentially determined by its tissue of origin. In this regard, K19 downregulation in breast cancer cells is shown to increase cell proliferation and migration. Here, K19 functions as a tumor repressor by downmodulating AKT signaling (Ju *et al.* 2013). Our work on the K8/K18 pair showed that this pair may play a significant role in modulating $\alpha 6\beta 4$ integrin-mediated signaling to modulate phenotypes like tumorigenicity, cell motility and cell invasion which collectively contribute to human oral tumor progression. In this study, the downregulation of K8 in an OSCC-derived cell line resulted in the downregulation of $\alpha 6\beta 4$ integrin levels, downstream effectors of $\beta 4$ integrin-mediated signaling, and an actin-binding protein, fascin (Alam *et al.* 2011b). In mixed epithelia like breast, we see an exactly reverse role played by K8/K18. Here, we have demonstrated that the downregulation of the K8/K18 pair in MDAMB468, a noninvasive cell line derived from breast carcinoma, leads to an increase in cancer cell migration, *in vitro* invasion, and anchorage-independent growth (Iyer *et al.* 2013). This once again highlights the context-dependent role of keratins and their ability to function differently in different cell/tissue type. The downregulation of K8/18 is also shown to activate the PI3K/Akt/NF-κB

pathways in epithelial cancer cell lines. Furthermore, the authors have also reported the cisplatin-sensitive phenotype of the K8/K18 depleted cells, which has been attributed to the increased cisplatin-induced apoptosis mediated by increased Fas receptor targeting on the membrane (Fortier *et al.* 2013). Furthermore, not only keratin expression but also different PTMs, especially phosphorylation on keratins, are shown to regulate diverse phenotypes in a cancer cell. In addition, evidence of keratin binding to heat-shock proteins also suggests that they have a certain role to play in controlling the stress and homeostatic balance of the cell. For instance, co-immunoprecipitation experiments using bladder cancer cells showed an interaction between Hsp74 and K1 in the cytoplasmic compartment (Chen *et al.* 2014). It will be interesting to investigate the phenotype and/or mechanism regulated by the interaction between Hsp74 and K1. Additionally, K8 is also shown to be present on the extracellular surface of breast cancer, hepatocellular cancer, and prostate cancer cells. In prostate cancer cells, extracellular K8 (eK8) was shown to associate with plasminogen to result in the degradation of vitronectin (ECM component of the prostate). This mechanism may be useful for the efficient dissemination of tumor cells *in vivo*. Along with this, the expression of eK8 can be used for the prognostication of prostate cancer (Kuchma *et al.* 2012). On the other hand, keratins are characteristic to a particular epithelium, and loss of keratins is associated with the appearance of epithelial mesenchymal transition (EMT). However, mice lacking keratin cytoskeleton (conditional deletion of keratin multifamily) failed to show any change with respect to EMT-related phenotypes like invasion, migration, proliferation etc., in a KRAS-driven murine lung cancer model (Konig *et al.* 2013). This suggests that loss of keratins occurs downstream of EMT induction and may not be responsible in driving the process of EMT. Besides the fact that keratins regulate several processes involved in tumor progression, there is also some literature available about what regulates keratins. On these lines, sirtuin2, which belongs to the sirtuin family of proteins known to be the sensors of metabolic, oxidative and genotoxic stress, is shown to regulate the expression of K19 in skin cancers. K19 is a stemness-related marker in case of skin epithelia and deletion of sirtuin4 led to the upregulation of K19, indicating that perhaps in the normal scenario, sirtuin4 suppresses the expression of K19 to promote the differentiation of the skin cells (Ming *et al.* 2014). Besides this, an interesting observation from our laboratory has also thrown some light on the regulators of keratins in cancer cells. We have shown that vimentin, which is a type III IF protein, is able to regulate the expression of K5/K14 in OSCC-derived cells. Here, the downregulation of vimentin led to a decrease in the expression of both K5 and K14 and the depletion of K5/K14 was strongly associated with the more differentiated phenotype of the cancer cell (Dmello *et al.* 2017).

Thus, keratins are now perceived as potential regulators, rather drivers of the cancer progression. Targeting of keratins and/or pathways regulated by them can be considered for diagnosis, prognosis and treatment of epithelial cancers. Furthermore, the identification of high-risk potentially malignant lesions can be carried out using keratins once large-scale follow-up studies on keratins in premalignant tissues are available.

6.4 *Keratins as determinants of drug responsiveness*

A strong association of keratins with transformation and progression of cancer makes them even more likely to be a drug target. For it to be a drug target, it is very essential to understand as to the presence or absence of which keratin confers resistance or sensitivity to a particular cell type. Parekh *et al.* had demonstrated that lower expression of K18 in ovarian adenocarcinoma cells is associated with resistance to cisplatin while higher expression of K18 is associated with sensitivity to cisplatin (Parekh and Simpkins 1995). However, K5 overexpression was associated with resistance to chemotherapy and poor prognosis in serous ovarian cancer patients. Hence, K5 overexpression could be used to identify resistant patients or could be targeted in order to improve patient survival (Ricciardelli *et al.* 2017). The differential proteomics-based studies using breast cancer tissues after neoadjuvant therapy showed the upregulation of K19, while K9 was downregulated in the resistant group. This study was able to categorize patients into the sensitive or the resistant group based on the differential expression of certain keratins along with other proteins like actin, HSP27, vimentin etc. (Yi *et al.* 2013). K10 expression is differentially regulated by the phosphatase and tensin homolog (PTEN) under the influence of cisplatin. This was demonstrated by the overexpression of PTEN in the ovarian cancer cell line, in the presence or absence of cisplatin. It appears that PTEN confers sensitivity to cisplatin through the upregulation of K10 (Wu *et al.* 2014). Furthermore, the overexpression of either PTEN or K10 was able to enhance cisplatin-induced inhibition of proliferation and apoptosis in ovarian cancer cells C13K and inhibit tumor growth of C13K xenografts (Wu *et al.* 2015).

Overall, it appears that keratins play a significant role in either conferring resistance or sensitivity to a particular drug, depending upon their degree of expression in a cancer cell of a specific cell type (figure 4). Therefore, in future, the expression of keratins may be envisaged to be useful in deciding the treatment modality or can be used as a treatment to sensitize a particular tumor.

7. Role of keratin phosphorylation in cancer

The biology of cancer is characterized by various hallmarks including uncontrolled cell proliferation, deregulation of apoptosis and increased cell migration. As we have

documented earlier in this review, cell migration is needed for tissue invasion and metastasis. Tumor progression is associated with dedifferentiation and loss of growth control, which have been linked to altered keratin phosphorylation in epithelial carcinomas.

Several reports support an active role of keratins as versatile regulators in carcinogenesis. However, reports regarding the role of keratin phosphorylation in carcinogenesis and metastasis are inconsistent. For example, an earlier report from our laboratory showed that the depletion of K8 phosphorylation in oral SCC-derived AW13516 cells leads to a more aggressive phenotype. In this study, Alam *et al.* showed that K8 dephosphorylation leads to an increase in cell migration and *in vivo* tumorigenicity. These observations suggested that K8 dephosphorylation provides a more aggressive phenotype to AW13516 cells. In concordance with this, they could also show loss of K8S73 and K8S431 phosphorylation in human OSCC. The dephosphorylation of K8 was significantly associated with size, stage, and lymph node metastasis and thereby the progression of the tumor (Alam *et al.* 2011a). In agreement with these findings, in another study, Mizuuchi *et al.* demonstrated that PRL-3 (phosphatase of regenerating liver 3), which belongs to PRL protein tyrosine phosphatase family, associates with K8, thus resulting in the dephosphorylation of K8. PRL-3 is known to increase the metastatic potential of CRCs (Mizuuchi *et al.* 2009). In addition, they observed an upregulation of PRL-3 and concomitantly more dephosphorylation of K8-S73 and K8-S431 at the invasive front of primary human CRC tissue samples. Altogether, they indicated an indirect role of K8 phosphorylation in the metastatic potential of CRC through PRL-3. In accordance with this, another study by Khapare *et al.* showed that depletion of the desmosomal plaque protein plakophilin3 (PKP3) in the HCT116 cell results in the increased oncogenic potential of these cells, together with an increase in K8 levels and a concomitant reduction in PRL-3 levels (Khapare *et al.* 2012). They hypothesize that the dephosphorylation of K8 through PRL-3 in PKP3-knockdown clones leads to the stabilization of K8 filaments, which affects tumor progression and metastasis in HCT116 cells. In summary, these reports suggested a correlation between K8 dephosphorylation and tumor progression and aggressiveness.

On the other hand, according to a previous report by Beil *et al.* sphingosylphosphorylcholine (SPC), a bioactive lipid that is elevated in blood and ascites of ovarian cancer patients, induced a perinuclear reorganization of keratin proteins via the phosphorylation of Ser-431 of K8, leading to increased migration of human pancreatic cancer cells (Beil *et al.* 2003). Furthermore, Busch *et al.* demonstrated that JNK and ERK phosphorylate K8 at Ser-431 and stimulate the perinuclear reorganization of keratin, resulting in enhanced migration. They have argued that both cell invasion through a connective tissue and its migration require the presence of a flexible leading edge (Busch *et al.* 2012). Along the same line, another report by Suresh *et al.* (2005) suggested that increased keratin phosphorylation might speed up the keratin cycling, thereby increasing network plasticity (Suresh *et al.* 2005). This might induce cell shape changes that are helpful for cell migration and invasiveness. This change in the keratin network architecture results in increased cellular elasticity and enhanced cell migration, indicating that SPC-induced keratin remodeling may directly contribute to the metastatic potential of epithelial cancer cells. Moreover, Rolli *et al.* (2010) indicated that cell deformability is also increased in association with keratin network alterations owing to SPC, likely resulting in greater ability of the cancer cell to invade the surrounding tissue and permeate through the stroma, thus facilitating its escape from the primary tumor (Rolli *et al.* 2010). Another study revealed the detailed mechanism of SPC-induced K8 phosphorylation and reorganization in cancer, suggesting that SPC induces EMP2 downregulation that reduces the PP2A via ubiquitination induced by cav-1, which sequestered α4 integrin, leading to the activation of ERK and JNK (Lee *et al.* 2016).

In concordance with this, Sec8 (exocyst complex component) has been acknowledged as an upstream regulator of ERK- and p38-mediated phosphorylation of K8 in migrating OSCC-derived HSC3 cells by Tanaka *et al.* Sec8 has been associated with several biological pathways like cell migration, invadopodia formation, cytokinesis, glucose uptake and neural development (Tanaka and Iino 2015). According to this study, loss of Sec8 in HSC3 cells inhibited their migration potential by affecting K8 phosphorylation at the Ser73 residue. This suggested a possible role of K8 dephosphorylation in reduced cell motility. A recent report from our laboratory has supported these findings, indicating that K8 dephosphorylation provides a less aggressive phenotype to skin epidermoid carcinoma-derived A431 cells. Our proteomic analysis identified a number of differentially regulated signaling pathways associated with biological functions like cell proliferation, migration, invasion and metastasis. In concordance with our proteomic data, we observed a significant reduction in the cell migratory, invasive and proliferative potential of the cells expressing K8 phospho-dead mutants (both K8S-73A and K8S431A). These observations further proved that K8 phosphorylation imparts increased aggressiveness to skin-SCC derived cells (Tiwari *et al.* 2017). Together, these reports substantiate the role of K8 phosphorylation in cancer progression in a context-dependent manner. *In vivo* animal model studies are required to prove the exact role of K8 phosphorylation in tumor progression.

Apart from K8, K18 phosphorylation has also been correlated with autophagy and apoptosis regulation of HCT116, colon carcinoma derived cells, under the effect of oxaliplatin, which is used in the treatment of colon and rectal cancers (Yan *et al.* 2016). Apart from this, the phosphorylation of K19-Y391 by an oncogenic kinase Src is shown to move its equilibrium towards the soluble fraction upon pervanadate treatment. Along with this, EGF treatment

which leads to Src activation is shown to stimulate oncogenesis by facilitating the Src and K19 interaction, indicating that K19 phosphorylation plays a critical role in carcinogenesis (Zhou *et al.* 2010). Various phosphatases involved in the dephosphorylation of keratins are implicated in the process of EMT, e.g. PRL-3 or PTP1B induce EMT, whereas PP2A, DEDD, and AMPK reverse EMT (Kim *et al.* 2015). Conclusively, phosphorylation of K8, 18 and 19 in the progression of different cancer types revealed its regulatory role in the various pathways. If investigated carefully in future, using animal models and human tumor tissue samples, they can serve as potential therapeutic targets. Apart from this, phosphorylation-associated function of other keratins such as K6, K17 etc. has not yet been explored but can be an interesting topic to study in future.

8. Concluding remarks

The progress made over the last two decades in the field of keratin biology clearly indicates that keratins are not merely structural proteins but also regulate many physiological processes by governing a number of signaling pathways. Keratins are unique in a way because they are K7 diverse, abundant, exhibit characteristic heteropolymerization ability with their specific binding partners and interact with a plethora of molecules in their milieu. This could possibly confer them with the ability to regulate unique yet multiple processes in a context-dependent fashion. Further research is necessary to have a better understanding of the assembly, dynamics and turnover of these proteins and their interactions with other proteins to decipher their context-dependent functions in the process of tissue homeostasis, cell transformation and tumor progression. This will help in establishing their worth as therapeutic targets. Also, a large-scale and a long-term follow-up study on human tumors is needed to establish the importance of keratins as prognostic markers.

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