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Review
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Phosphorylation and Reorganization of Keratin Networks: Implications for Carcinogenesis and Epithelial Mesenchymal Transition

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Abstract

Metastasis is one of hallmarks of cancer and a major cause of cancer death. Combatting metastasis is highly challenging. To overcome these difficulties, researchers have focused on physical properties of metastatic cancer cells. Metastatic cancer cells from patients are softer than benign cancer or normal cells. Changes of viscoelasticity of cancer cells are related to the keratin network. Unexpectedly, keratin network is dynamic and regulation of keratin network is important to the metastasis of cancer. Keratin is composed of heteropolymer of type I and II. Keratin connects from the plasma membrane to nucleus. Several proteins including kinases, and protein phosphatases bind to keratin intermediate filaments. Several endogenous compounds or toxic compounds induce phosphorylation and reorganization of keratin network in cancer cells, leading to increased migration. Continuous phosphorylation of keratin results in loss of keratin, which is one of the features of epithelial mesenchymal transition (EMT). Therefore, several proteins involved in phosphorylation and reorganization of keratin also have a role in EMT. It is likely that compounds controlling phosphorylation and reorganization of keratin are potential candidates for combating EMT and metastasis.

Key Words: Metastasis, Viscoelasticity, Phosphorylation of keratin, Reorganization of keratin, Epithelial Mesenchymal Transition, Sphingosylphosphorylcholine

INTRODUCTION

Metastasis is critical hallmark of cancer and contributes to the 90% of cancer death (Hanahan and Weinberg, 2011). Diverse approaches have been attempted to combat the metastasis of cancer. The spot light has been on matrix metalloproteinase inhibitors but the clinical outcome of matrix metalloproteinase inhibitors in most cancer metastasis is poor (Coussens *et al*., 2002; Pavlaki and Zucker, 2003).

Recently, several researchers investigated physical properties of cancer cells and found that metastatic cancer cells are significantly softer than other benign or normal cells (Cross *et al*., 2007). This softness of metastatic cancer cells might be useful as diagnostic marker. Measures of physical properties might also be useful as assay methods for new compounds modulating the physical properties of cancer cells using novel devices such as optical stretcher, optical tweezer, and atomic force microscopy (Suresh, 2007).

Because the physical properties and mechanotransduction of cancer cells are crucial in various steps of the metastatic process, control of physical properties of cancer cell may be an effective therapeutic approach for patients suffering cancer (Stroka and Konstantopoulos, 2014).

However, measuring changes of physical properties of cancer cells is not easy to most researchers in pharmacology fields. We are interested in the biological phenomena reflecting the changes of physical properties such as keratin reorganization via phosphorylation, which is changed by sphingosylphosphorylcholine (SPC) and related to viscoelasticity of metastatic cancer cells (Beil *et al*., 2003). We have studied the underlying molecular mechanisms in keratin 8 (K8) phosphorylation and perinuclear reorganizations of cancer cells for several years. We have reviewed the results of these studies together with the relevant literature.

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Table 1. Expression of keratin proteins in epithelial tissues*

Keratin	Epithelial tissue	Partner
Type I		
Simple		
K18	Simple epithelia (e.g. liver, pancreas, colon, lung)	K8, K7
K20	Simple epithelia, especially gastrointestinal	K8, (K7)
Barrier		
K9	Stratified cornifying epithelia; palm, sole	(K1)
K10	Stratified cornifying epithelia; suprabasal	K1
K12	Stratified epithelia; cornea	K3
K13	Stratified epithelia; non-cornifying; suprabasal	K4
K14	Stratified and complex epithelia; basal	K5
K15	Stratified epithelia	(K5)
K16	Stratified epithelia; induced during stress, fast turn over; suprabasal	K6a
K17	Stratified epithelia; induced during stress, fast turn over	K6b
K19	Simple and stratified epithelia	K8
K23, K24	Epithelia	
Structural		
K25, K26, K27, K28	Stratified epithelia; hair follicle sheath	
K31, K32, K33a, K33b, K34, K35, K36, K37, K38, K39, K40	Stratified epithelia; hair, hard structure	
Type II		
Simple		
K7, K8	Simple epithelia	K18
Barrier		
K1	Stratified cornifying epithelia; suprabasal	K10
K2	Stratified cornifying epithelia; late suprabasal	(K10)
K3	Stratified epithelia, cornea	K12
K4	Stratified epithelia; non-cornifying; suprabasal	K13
K5	Stratified and complex epithelia; basal cells	K14, (K15)
K6a	Stratified epithelia; induced during stress, fast turn over	K16
K6b	Stratified epithelia; induced during stress, fast turn over	K17
K6c	Epithelia	
K76	Stratified cornifying epithelia, oral, suprabasal	(K10)
K78, K79, K80	Epithelia	
Structural		
K75	Stratified epithelia; hair follicle	
K71, K72, K73, K74	Stratified epithelia; hair follicle sheath	
K81, K82, K83, K84, K85, K86	Stratified epithelia; hair, hard structure	

*Modified from Haines and Lanes, and Loschke (Haines and Lane, 2012; Loschke *et al*., 2015).

STRUCTURE AND CHARACTERISTICS OF KERATINS

Epithelial cell keratins are composed of heteropolymer of one type I keratin and one type II keratin proteins (Table 1) (Coulombe and Omary, 2002). Keratin contains a common α-helical rod domain of ~310 amino acid, sided by non-helical head and tail domains of diverse length and sequence having several phosphorylation sites (Ku *et al*., 1998; Omary *et al*., 2006; Loschke *et al*., 2015) (Fig. 1).

Simple epithelia of liver, intestine, and pancreas, are discovered as pairs of K7, K8, K18, K19, and K20, but the ratio of type I and type II keratins is 1:1 in all cells (Moll *et al*., 1982; Ku *et al*., 1999; Toivola *et al*., 2002). K8 and K18 assemble to form heterodimers in epithelia of gland (Omary *et al*., 2009; Toivola *et al*., 2015). Keratins assemble as heterodimers of each of type I and type II keratin monomer, aligned in parallel (Hatzfeld and Weber, 1990; Herrmann and Aebi, 2000; Haines and Lane, 2012). These heterodimers convert to antiparallel tetramers by overlaying the N-terminal half of rod domains and tetramers then form 'unit length filaments' (60 nm in length) (Fig. 2) (Haines and Lane, 2012).

Several situations including diverse stress requires the changes of keratins (Leube *et al*., 2011). Keratin cycle starts with nucleation of keratin units at the peripheral region of cells including vicinity of focal adhesions (Windoffer *et al*., 2011). Next, elongation of new keratin units follows actin-dependent movement toward the peripheral keratin network (Windoffer *et al*., 2011). After consolidation of keratin particles to the keratin network, keratin filaments keep to move toward the rim of nucleus and bundle (Loschke *et al*., 2015). Parts of keratins break up into several pieces of oligomers that diffuse into the cytosol (Loschke *et al*., 2015). Other keratins make a peri-

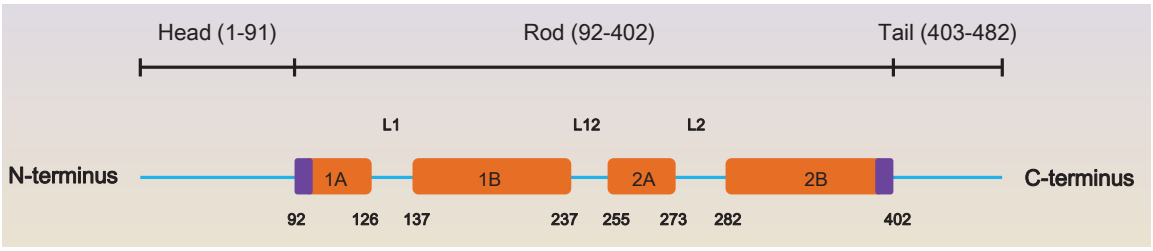

Fig. 1. Domain Structure of keratin 8. Keratin proteins are composed of the non-helical N-terminal head- and C-terminal tail-domains as well as the in the middle helical rod-domain (Toivola *et al*., 2015). The 4 α-helical parts (1A, 1B, 2A and 2B) of the rod domain are combined through the linker domains L1, L12 and L2. The number and domain shown here is K8 based on www.interfil.org. Modified from Toivola *et al*. (Toivola *et al*., 2015).

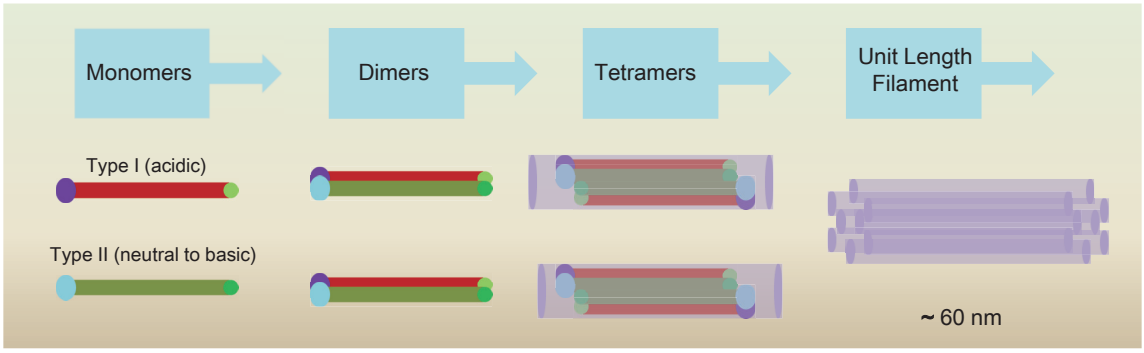

Fig. 2. Assembly of keratin filaments. The strands are made up of keratin filament proteins. Keratin filament proteins have the same basic structure: they have a globular head at their N-termini, a globular tail at their C termini, and a rod-like α helical domain in between (Haines and Lane, 2012). Two such units can twist each other to shape a "coiled coil" structure (Haines and Lane, 2012). Two of these coiled coils align head-to-tail to form a tetramer. Eight tetramers align end to end to form a unit length filament (32 monomer) (Haines and Lane, 2012). Modified from Haines and Lanel (Haines and Lane, 2012).

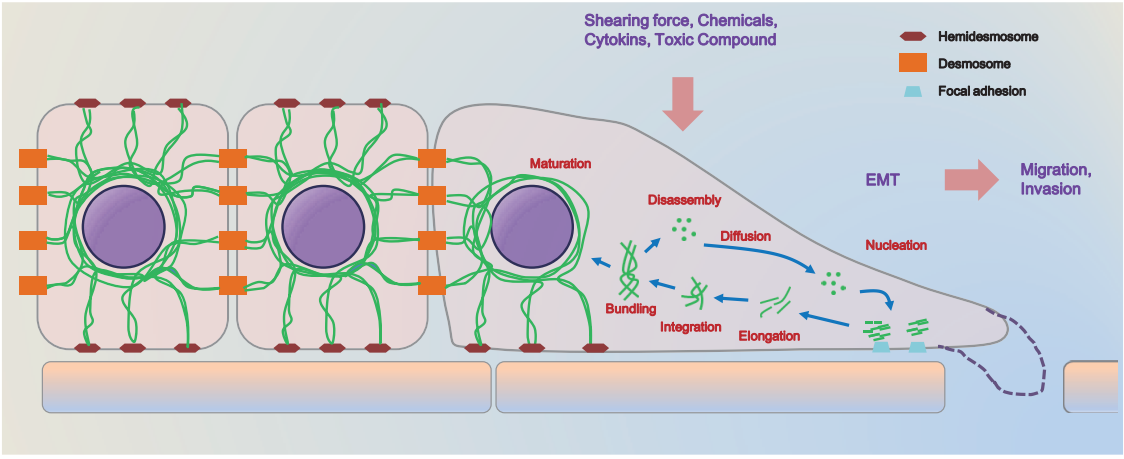

Fig. 3. The keratin cycle. Soluble keratin oligomers congregate into particles in the peripheral region of cells in proximity to focal adhesion sites (nucleation)(Windoffer *et al*., 2011). These particles grow (elongation) and move toward the cell center in an actin-dependent process (transport) (Windoffer *et al*., 2011). Subsequently, elongated keratin particles are combined into the peripheral keratin network (integration) (Windoffer *et al*., 2011). Filament bundling occurs during further centralizing translocation toward the nucleus (transport) (Windoffer *et al*., 2011). Soluble oligomers set apart (disassembly), diffuse throughout the cytoplasm (diffusion), and are recycled for another turn of keratins formation in the cell periphery (Haines and Lane, 2012). Alternatively, bundled keratin filaments are stabilized (maturation), making the stable perinuclear cage. Modified from Haines and Lanel (Windoffer *et al*., 2011; Haines and Lane, 2012).

nuclear keratin network and are linked to desmosome and hemidesmosome (Fig. 3) (Windoffer *et al*., 2011).

KERATIN IN THE EPITHELIAL CELLS

In the epithelia tissues, a network of proteins links the nu-

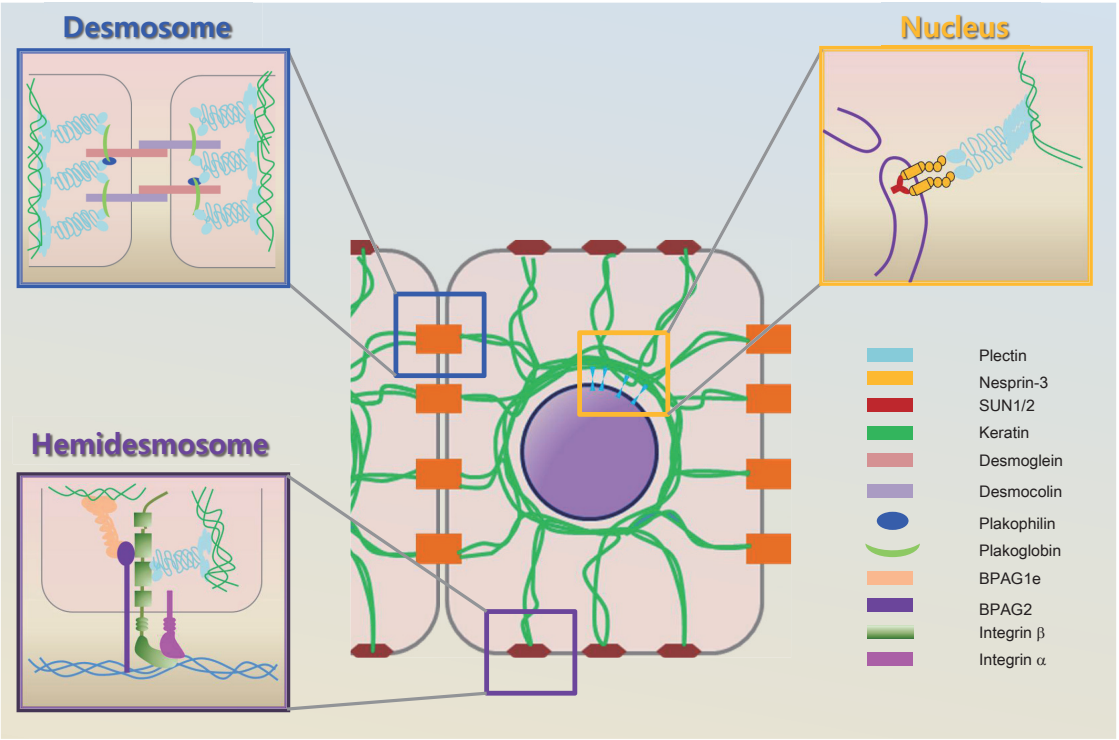

Fig. 4. Keratin in epithelial cells. Desmosome junction: Desmosomes link to the keratin filament of cells. Transmembrane desmosomal cadherins, desmoglein and desmocolin, bind placoglobin, the armadillo family protein, which holds the plectin, plakin family member (Fuchs and Raghavan, 2002). The cytoplasmic plaque anchors the keratin intermediate to the desmosome. Hemidesmosome junction: Integrin α and β heterodimers consist of the core of the hemidesmosome, along with BPAG2, a transmembrane protein. BPAG1e and plectin are two hemidesmosomal proteins that are members of the plakin family (Haines and Lane, 2012). They seem to function by connecting the keratin filament to the transmembrane proteins in the hemidesmosome. BPAG1e, bullous pemphigoid antigen 1, epidermal isoform; BPAG2, bullous pemphigoid antigen 2 (Haines and Lane, 2012). Nuclear junction: Nesprin 3 attach to SUN proteins through the perinucelar space and can directly connect to keratin proteins via plectin (Gerlitz and Bustin, 2011). Modified and combined from Fuchs and Raghavan, Gerlitz and Bustin, and Haines and Lanel (Fuchs and Raghavan, 2002; Gerlitz and Bustin, 2011; Haines and Lane, 2012).

cleus to membrane of cell through keratin filaments, in which transmembrane proteins gives the ground for cell to cell and cell to extracellular matrix adhesion (Fig. 4) (Omary *et al*., 2009; Pan *et al*., 2013).

Linking to desmosome and hemidesmosome

Keratin is connected to desmosome in the cell to cell adhesion site through desmoplakin (Green and Simpson, 2007). The cadherin family, the desmogleins and desmocollins, join the adhesion point (Getsios *et al*., 2004; Green and Simpson, 2007). The tails of the cadherins give an association region for the armadillo proteins such as plakoglobin, plakophilins 1-3, and p0071 (Schmidt and Jager, 2005; Green and Simpson, 2007). The carboxy terminal of desmoplakin interacts directly with the amino terminal end of type II keratins (Fig. 4) (Kouklis *et al*., 1994; Hatsell and Cowin, 2001).

Hemidesmosomes are junction complexes contributing to the adherence of epithelial cells to the basal layer (Borradori and Sonnenberg, 1999). The molecular structure of hemidesmosome is composed of 3 kinds of proteins: the cytoplasmic linker proteins for intermediate filaments at the cytoplasmic leaflet of the plasma membrane, the transmemebrane proteins acting as receptors linking the inside of cell to the proteins of the basal layers (Borradori and Sonnenberg, 1999). Keratin is linked to plectin and BPAG1e at hemidesmosome cell-matrix adhesions (Guo *et al*., 1995; Green and Simpson, 2007; Pan *et al*., 2013). The linking of plectin to keratins is required for hemidesmosome assembly (Fig. 4) (Koster *et al*., 2004). Keratins localize hemidesmosomes and repress migration of cells (Seltmann *et al*., 2013).

Linking to nucleat envelope

Lamins underlie the inner face of nuclear membrane and also make stable structures within the nucleus interior which contains emerin, lamin B-receptor, and SUN (Sad1 and UNC84 domain containing) 1/2 (Friedl *et al*., 2011). Nesprins belong to a family of proteins that are mainly known for their position along the nuclear envelope (Mellad *et al*., 2011).

Nesprins are a core member of the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex that cross over both nuclear membranes to link the cytoplasm and the inside of nucleus (Neumann and Noegel, 2014).

Nesprins interact with SUN proteins through perinuclear space via their KASH (Klarsicht, ANC-1, Syne Homology) domain and directly link to actin filaments (nesprin-1 and -2) and keratins via plectin (nesprin-3) (Padmakumar *et al*., 2005; Friedl *et al*., 2011). The cytoplasmic N-terminus of nesprin-3 interacts with plectin, a member of the plakin family of cytoskeletal linker protein (Sonnenberg and Liem, 2007). Nesprin-1 and -2 bind to microtubules via kinesin or dynein

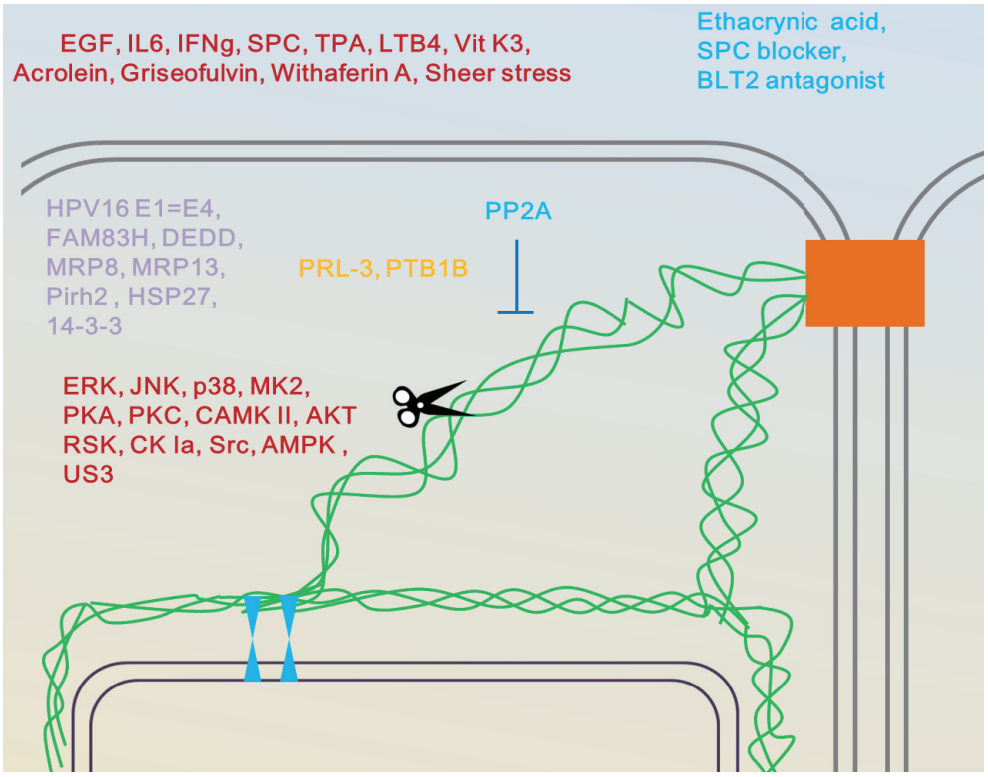

Fig. 5. Keratin networks as targets and effectors of chemical signals and stresses. Desmosomes locations at which growth factor signaling and force are sensed and transmitted, organize keratin networks (Haines and Lane, 2012). Posttranslational keratin modifications including several kinases and phosphatases enhance keratin network dynamics and the non-polymeric keratin state (Loschke *et al*., 2015). These modifications evoke weaker cell adhesion, enhanced migration and invasion of epithelial cells. Modified from Loschke *et al*., (Loschke *et al*., 2015).

(Friedl *et al*., 2011; Rajgor *et al*., 2014).

Linking to microfilaments & microtubules

Keratin particles emerge from the vicinity of the plasma membrane, maneuver continuously toward the central part of cell, and consolidate into the peripheral keratin network (Kolsch *et al*., 2009). These keratin cycles are highly dependent on interaction with actin filament (Pan *et al*., 2013). Actin depolymerization rapidly triggers keratin intermediate filament formation by turning on keratin related genes (Chang *et al*., 2014).

Keratin particles also moves fast via microtubules (Liovic *et al*., 2003). Keratin shows 2 types of motility in cells such as slow, continuous transport of keratin precursor particles of cell, and fast, bidirectional movement of keratin particles (Woll *et al*., 2005). Type I movement is mediated by actin and type II movement is mediated by microtubule systems (Woll *et al*., 2005).

Spectroplakins are big cytoskeletal linking proteins that bind to all 3 members of the cytoskeleton such as actin filaments, microtubules, and intermediate filaments (Suozzi *et al*., 2012). The spectraplakin family is composed of two mammalian genes, MACF1 (Microtubule-Actin Crosslinking Factor 1), and Dst (Dystonin) encoding bullous pemphigoid antigen 1 (Suozzi *et al*., 2012). BPAG1 connects the keratin network to hemidesmosome of cell to intensify the mechanical strength at the basal layer of the epidermis (Koster *et al*., 2003; Suozzi *et al*., 2012).

PHOSPHORYLATION OF KERATINS

A wide range of post-translational modifications have been reported on keratins such as phosphorylation, ubiquitylation, acetylation, glycosylation, and, sumoylation, which seem to control the solubility of keratins in several situations (Omary *et al*., 2006; Ku *et al*., 2010; Srikanth *et al*., 2010; Snider *et al*., 2011). Recently, a review focuses on post-translational modification of intermediate filament proteins including vimentin and keratin (Snider and Omary, 2014). So we just emphasize phosphorylation of keratin which is key event in perinuclear reorganization of keratin (Beil *et al*., 2003).

Phosphorylation is a key reaction of keratins, and K1, K8, K18, and K19 are the fully studied among keratin family (Steinert, 1988; Zhou *et al*., 1999; Omary *et al*., 2002). Multiple factors such as several stresses, apoptosis, and mitosis, regulate keratin phosphorylation resulting keratin filament reorganization (Ku *et al*., 1999). Serine is the primary amino acid of phosphorylated keratin (Oshima, 1982; Omary *et al*., 1998). Tyrosine and threonine are also phosphorylated keratin residues (Feng *et al*., 1999). Sphingosylphosphorylcholine (SPC)-induced phosphorylation and perinuclear reorganization of keratin are implicated in viscoelasticity of PANC-1 cancer cells (Beil *et al*., 2003). Therefore, keratin phosphorylation seems

Table 2. Phosphorylated residues of keratins and kinases involved

Keratins	Phosphorylated residues	Kinases involved	References
K8	Ser-8	PKA, PKC_{ε}	(Akita *et al*., 2007; Ando *et al*., 1996)
	Ser-12	PKA	(Ando *et al*., 1996)
	Ser-23	PKA, PKC_{ε}	(Akita *et al*., 2007; Ando *et al*., 1996)
	Ser-33	PKA	(Ando *et al*., 1996)
	Ser-36	PKA	(Ando *et al*., 1996)
	Ser-42	PKA	(Ando *et al*., 1996)
	Ser-50	PKA	(Ando *et al*., 1996)
	Ser-73	JNK, PKC_{δ}, MK2*	(He *et al*., 2002; Menon *et al*., 2010; Ridge *et al*., 2005)
	Ser-416	PKA	(Ando *et al*., 1996)
	Ser-423	PKA	(Ando *et al*., 1996)
	Ser-425	PKA	(Ando *et al*., 1996)
	Ser-431	ERK, JNK	(Busch *et al*., 2012; Park *et al*., 2011; Park *et al*., 2012)
	Not determined	AKT, AMPK, CAMK II, CK-Iα,	(Kuga *et al*., 2013; Loschke *et al*., 2015; Velasco *et al*., 1998; Yano *et al*., 1991)
K17	Ser-44	RSK1	(Pan *et al*., 2011)
	Not determined	US3	(Murata *et al*., 2002)
K18	Ser-33	PKC_{ζ}	(Sivaramakrishnan *et al*., 2009)
	Ser-52	MK2	(Menon *et al*., 2010)
	Not determined	AMPK	(Velasco *et al*., 1998)
K19	Ser-35	Not determined	(Zhou *et al*., 1999)
	Tyr-391	Src kinase	(Zhou *et al*., 2010)
K20	Ser-13	MK2, PKC*	(Menon *et al*., 2010; Zhou *et al*., 2006)

*No evidence for phosphorylation of residue by indicated kinase but dependent on that.

to be important in regulating the physical properties of cancer cells. However, it is not yet clear that perinuclear reorganization by phosphorylation is a special event for metastatic cancer or just one step of keratin recycle process. In addition, it is not clear why metastatic cancer cells reveal phenotypes such as the perinuclear reorganized keratin structure.

PLAYERS INVOLVED IN PHOSPHORYLATION AND REORGANIZATION OF KERATINS

Mitogen-activated protein (MAP) kinases

Numerous kinases are involved in phosphorylation of keratins (Snider and Omary, 2014). Phosphorylation of serine residue of keratin leads to disintegration of the stable structure and increased solubility of keratin in the cytoplasm (Omary *et al*., 1998).

ERK is one of the kinases involved in SPC or leukotriene B4 (LTB4)- evoked K8 phosphorylation and reorganization (Fig. 5, Table 2) (Busch *et al*., 2012; Park *et al*., 2012). ERK is also required in acetone extracts from *Bupleurm scorzonerifolium*-induced K8 phosphorylation in A549 cancer cells (Chen *et al*., 2005).

Serine-73 (Ser-73) of K8 is a residue of phosphorylation by c-Jun N-terminal kinase (JNK) (Fig. 5, Table 2). Furthermore, we found that JNK phosphorylates serine-431 (Ser-431) in SPC-induced phosphorylation and reorganization of K8 (He *et al*., 2002; Park *et al*., 2011).

p38 mitogen activated protein kinase (MAPK) is also involved in phosphorylation of Ser-73 induced by treatment with okadaic acid or orthovanadate (Ku *et al*., 2002a; Woll *et al*., 2007). p38 MAPK phosphorylates MAPK-activated protein kinase MK2 and phosphorylation of Ser-73 in HT29 cells is dependent on MK2 (Fig. 5, Table 2) (Menon *et al*., 2010). MK2 also phosphorylates Ser-52 of K18 and Ser-13 of K20 (Menon *et al*., 2010).

PKA, PKC, and CAMK II

cAMP-dependent protein kinase (PKA) and Ca^{2+}- dependent protein kinase C (PKC) almost exclusively phosphorylates serine of K8 (Fig. 5, Table 2) (Yano *et al*., 1991). PKA phosphorylates Ser-8, Ser-12, Ser-23, Ser-33, Ser-36, Ser-42, and Ser-50 in the head domain and Ser-416, Ser-423, and Ser-425 in the tail region of K8 (Ando *et al*., 1996). Protein kinase C_{ε} (PKC_{ε}) phosphorylates K8 at Ser-8 and Ser-23 in thyrotropin-releasing hormone (TRH) -treated GH4C1 cells (Akita *et al*., 2007). Interestingly, PKCε and K8 have perinuclear colocalization under basal conditions and are found in the cell periphery and cell to cell contact region after TRH treatment (Akita *et al*., 2007). Protein kinase C_{δ} (PKC_{δ}) phosphorylates Ser-73 of K8 regulating the shear stress-mediated collapse of keratin network in human A549 cells (Ridge *et al*., 2005). Protein kinase C_{ζ} (PKC_{ζ}) phosphorylates Ser-33 of K18 leading to reorganization of keratin proteins induced by shear stress (Sivaramakrishnan *et al*., 2009). Phosphorylation of Ser-13 of K20 is increased after PKC activation but it is not clear whether PKC phosphorylates Ser-13 of K20 (Zhou *et al*., 2006). Recently, K8 phosphorylation by PKC is known to a major contributing factor for K8 downregulation in human disc degeneration (Sun *et al*., 2013).

Calmodulin-dependent protein kinase II (CAMK II) phosphorylates K8 at serine and threonine amino acids (Yano *et al*., 1991). However, specific sites of phosphorylated residues of K8 by CAMK II were not reported.

AKT and RSK

Predicted phosphorylation sites for Akt exist in several keratins and Akt binds K8 but not K18 (Fig. 5, Table 2) (Paramio *et al*., 2001; Loschke *et al*., 2015). In the presence of K8 and K18, K8-Akt interaction is independent of K18 glycosylation and Thr 308-phosphorylation in Akt1 (Ku *et al*., 2010). Akt1 overexpression also increases K8 and K18 proteins (Fortier *et al*., 2010b). However, there are no reports on Akt-induced phosphorylation of specific residue(s) of keratin.

K17, a type I keratin, is heavily induced in epidermis after injury, and in psoriasis and cancer (Pan *et al*., 2011). p90 ribosomal protein S6 kinase 1 (RSK1) phosphorylates Ser-44 residue of K17 of keratinocytes (Fig. 5) (Pan *et al*., 2011). However, this phosphorylation is not clearly linked to a modification of keratin network.

Casein kinase Iα

Casein kinase Iα (CK-Iα) plays an essential role in the phosphorylation and degradation of β-catenin (Knippschild *et al*., 2005). Casein kinase Iα (CK-Iα) mediates FAM83H (family with sequence similarity 83 member H)-dependent reorganization of keratin filaments (Kuga *et al*., 2013). Inhibition of CK-1α is a cause of keratin filament bundling and reverses keratin filament disassembly; but it is not yet known which amino acid residue of K8 or K18 is phosphorylated by CK-Iα. Ser-73 and Ser-431 of K8 and Ser-33 and Ser-52 of K18 are not candidates of substrates of CK-Iα (Fig. 5, Table 2) (Kuga *et al*., 2013).

Src kinase

Ser-35 of K19, which is a type I keratin, is a well-known residue of phosphorylation (Zhou *et al*., 1999). Src kinase phosphorylates tyrosine 391 of human K19 (Fig. 5, Table 2) (Zhou *et al*., 2010). During keratinocytes migration and tissue repair, Src kinase activity is inhibited by wound-induced keratin such as K6a and K6b (Rotty and Coulombe, 2012).

Miscellaneous kinases

The AMP-activated protein kinase (AMPK) is important in the biological response induced by metabolic changes and is turned on by AMP (Velasco *et al*., 1998). AMPK and 5-aminoimidazole-4-carboxamide ribonucleotide, a AMPK activator phosphorylate K8 and K18 in primary hepatocytes (Fig. 5, Table 2) (Velasco *et al*., 1998).

US3 is a specific serine/threonine protein kinase found in herpes simplex virus (Murata *et al*., 2002; Koyanagi *et al*., 2014). US3 protein kinase directly phosphorylates K17 (Fig. 5, Table 2) (Murata *et al*., 2002). However, there are no reports on US3 or AMPK-induced phosphorylation of specific residue(s) of keratin.

OTHER PLAYES IN KERATIN PHOSPHORYLATION AND REORGANIZATION

Protein phosphatase

Several kinases are reportedly implicated in the SPC-induced phosphorylation of K8. For example, ERK and JNK are involved in SPC-induced K8 phosphorylation (Park *et al*., 2011; Busch *et al*., 2012). So common upstream regulator of ERK and JNK might be important in SPC-induced K8 phosphorylation. Protein phosphatase-2A (PP2A) dephosphorylated phospho ERK and phospho JNK (Fig. 5) (He *et al*., 2002; Hu *et al*., 2009). PP2A directly dephosphorylates K8 during hyposmotic stress in HT29 cells (Tao *et al*., 2006). PP2A also maintains the structure and interactions of hepatic keratin intermediate filaments (Toivola *et al*., 1997). PP2A down regulation is also involved in LTB_4-evoked phosphorylation of K8 at Ser-431 (Park *et al*., 2012).

Phosphatase of regenerating liver-3 (PRL-3) belongs to the PRL protein tyrosine phosphatase family and highly PRL-3 expressed cancer cells demonstrate reduction of K8 phosphorylation, especially at the front of invasion and metastasis to liver (Fig. 5) (Mizuuchi *et al*., 2009). Especially, loss of plakophilin 3 results in an increase in PRL3 levels promoting K8 dephosphorylation of HCT116 cells (Khapare *et al*., 2012).

Pharmacological inhibition of the protein-tyrosine phosphatase PTP1B increases phosphorylation of Tyr-267 of K8, decreases solubility, and increases K8 filament bundling, whereas PTP1B overexpression has the opposite effects (Fig. 5) (Snider *et al*., 2013).

It seems that effects on K8 structure and stability by phosphorylation of serine differ from those of tyrosine phosphorylation. Further study is needed to elucidate the role of different phosphorylated keratins on structure and reorganization.

Miscellaneous binding partner of keratins

High-risk human papillomaviruses (HPV) such as HPV16, are the major cause of cervical cancer and one of HPV16 proteins, E1-E4 binds to keratins leading to keratin network disorganization (Fig. 5) (Wang *et al*., 2004). Albatross exists with keratin filaments in nonpolarized epithelial cells and keratins stabilize the Albatross protein (Fig. 5) (Sugimoto *et al*., 2008). A newly identified keratin-associated protein, FAM83H regulates the filamentous state of keratins and the C-terminal region of FAM83H interacts with keratins (Fig. 5) (Kuga *et al*., 2013).

Death effector domain with DNA binding protein (DEDD), is present mostly as mono- or diubiquitinated form, and diubiquitinated DEDD bind to the K8 and K18 (Fig. 5) (Lee *et al*., 2002). Migration inhibitory factor-related protein 8 (MRP8) and MRP14, may be implicated in Ca^{2+}-induced keratins reorganization in TR146 human squamous cell carcinoma (Fig. 5) (Goebeler *et al*., 1995). p53-induced ubiquitin-protein ligase (Pirh2), binds to K8 and K18 and phosphorylation of either Pirh2 or K8 and K18, influences their binding (Fig. 5) (Duan *et al*., 2009).

Association of small heat shock proteins (HSP) with intermediate filament including keratins, may regulate filament interactions in cellular networks. For example, the chaperone HSP27 affects assembly dynamics and organization of K8 and K18 cytoskeleton through direct keratin interactions (Fig. 5) (Perng *et al*., 1999; Kayser *et al*., 2013; Loschke *et al*., 2015).

14-3-3 protein binds to several kinases of signal transduction (Liao and Omary, 1996). 14-3-3 proteins also interact with phosphorylated form of keratin in simple epithelia during the course of cell cycle and plays a role of cofactor for solubilization of keratins (Liao and Omary, 1996). Ser-33 phosphorylation of K18 influences binding of K18 to 14-3-3 proteins in the course of mitosis and interaction of K18 with 14-3-3 proteins regulates keratin filaments and mitotic progression of hepatic cells (Fig. 5) (Ku *et al*., 2002b).

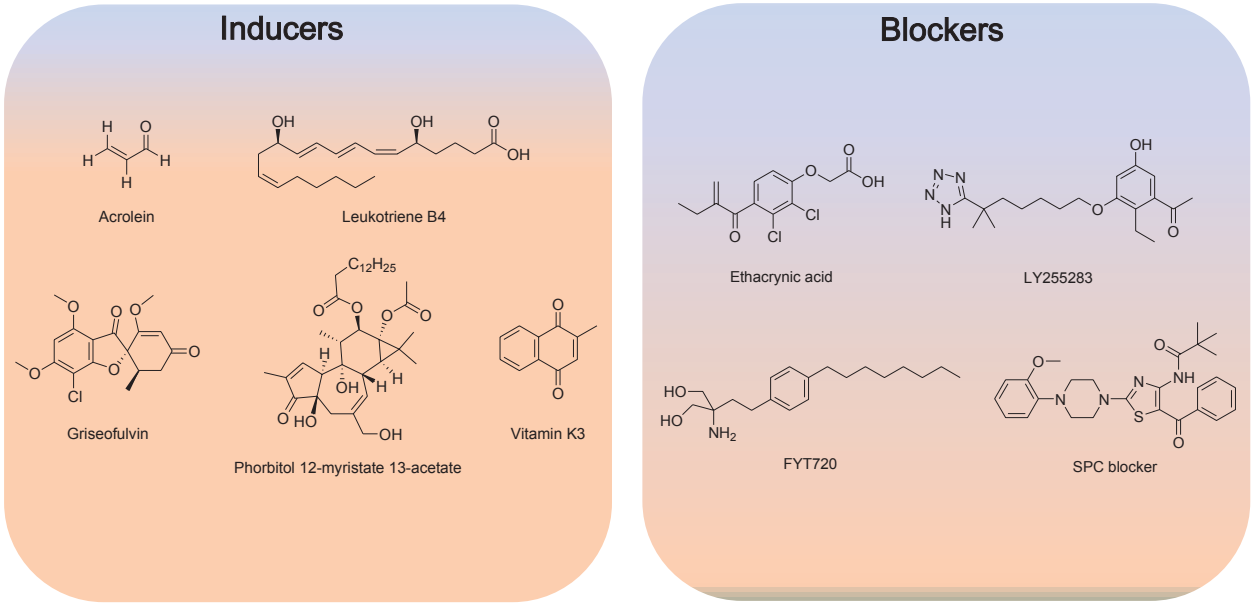

Fig. 6. Structure of inducers and blockers of keratin phosphorylation and reorganization.

INDUCERS OF PHOSPHORYLATION AND REORGANIZATION OF KERATINS

Growth factor & cytokines

Epidermal growth factor (EGF) leads to phosphorylation of keratin in rat hepatocyte before keratin reorganization (Fig. 5) (Baribault *et al*., 1989). EGF-induced K8 phosphorylation happens at Ser-23 of head domain and Ser-431 of tail domain (Ku and Omary, 1997).

Interleukin-6 (IL-6) significantly up-regulates K8 and K18 in intestinal epithelial cells such as Caco2-BBE (brush border expressing) cell line and IL-6 evoked K8 phosphorylation at serine residue (Fig. 5) (Wang *et al*., 2007b). IL-6 protect intestinal barrier via K8/K18 in compromised condition (Wang *et al*., 2007b).

K17, the myoepithelial keratin, is expressed in psoriasis but is not present in healthy skin (Komine *et al*., 1996). Increased production of interferon gamma (IFNγ) induces the expression of K17 by activating transcription factor STAT1 (Komine *et al*., 1996). However, it is not clear whether IFNγ induces phosphorylation and reorganization of K17.

12-O-Tetradecanoylphorbol-13-acetate & LTB_4

Exposure of the hepatocytes to 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (150 nM), a typical activator of protein kinase C, leads to phosphorylation of K8 but not K18 (Cadrin *et al*., 1992). Recently, we found that transglutaminase-2 plays important role in TPA-induced K8 phosphorylation and reorganization (Fig. 6) (Lee *et al*., 2014). Our data show that LTB_4 is an inducer of K8 phosphorylation and that ERK is involved in LTB_4-induced phosphorylation and reorganization of K8 in pancreatic cancer cells. LTB_4 receptor 2 (BLT2) receptor mediates effects of LTB_4 via PP2A down-regulation (Fig. 5) (Park *et al*., 2012).

Chemical compounds & others including physical stresses

Treatment of several human breast cancer cells including MCF7, T47D, SKBR3 with vitamin K3 (50-100 μM) leads to K8 phosphorylation at Ser-73 via MEK (MAPK/ERK kinase) 1/2 signaling (Fig. 6) (Scott *et al*., 2005).

Acrolein is a primary mediator of pulmonary edema and induces phosphorylation of K8 at Ser-73 in bronchiolar lung epithelia (Fig. 6) (Burcham *et al*., 2014).

Griseofulvin induces Mallory-Denk bodies in hepatocytes of mice (Fortier *et al*., 2010a). In this mice model, griseofulvin induces phosphorylation of K8 (Ser-79, Ser-436) and K18 (Ser-33) (Fig. 6).

Pervanadate, tyrosine phosphatase inhibitor, induces phosphorylation of tyrosine residue in K8, and K19, but not K18 via p38 MAP kinase (Feng *et al*., 1999). This process appears independent of ERK kinase pathway.

Withaferin A (WFA) binds to the vimentin and modifies perinuclear aggregates of intermediates filaments including keratin (Grin *et al*., 2012).

Compressive loads induce K8 phosphorylation in human disc generation by activating protein kinase C (Sun *et al*., 2013). Shear stress also evokes reorganization of the keratin network via the phosphorylation of K8 by PKC_ζ (Sivaramakrishnan *et al*., 2009). Heat stress or rotavirus infection induced phosphorylation of K8 in human colonic cell line HT29 (Liao *et al*., 1995).

PHOSPHORYLATION AND REORGANIZATION OF KERATINS IN CANCER

Several reports support an active role of keratins as versatile regulators in carcinogenesis (Karantza, 2011). However, roles of phosphorylation of keratin in carcinogenesis and metastasis are controversial. For example, loss of K8 Ser-73 and Ser-431 phosphorylation is also observed in human oral squa-

mous cell carcinoma (OSCC) tissues evaluated by immunohistochemistry, in which dephosphorylation greatly associated with size, and progression of the tumor (Alam *et al*., 2011). Moreover, overexpression of K8 and K18 is related to up-regulation of histone type 2 H2aa3 and keratin reorganization may accelerate cancerous transformation of glutathione S-transferase P-form positive foci in the course of rat hepatocarcinogenesis (Kakehashi *et al*., 2009). Similarly, K19 expression in human hepatocellular carcinoma is correlated with increased invasiveness and metastasis (Govaere *et al*., 2014).

On the other hand, loss of K8 and K18 leads to increased collective emigration and invasiveness of breast cancer cells (Fortier *et al*., 2010a). Similarly, SPC evokes a perinuclear reorganization of keratin proteins via phosphorylation of Ser-431 of K8, and increased migration of human pancreatic cancer cells (Beil *et al*., 2003). JNK and ERK phosphorylates K8 at Ser-431, and stimulate the perinuclear reorganization of keratin resulting enhanced migration (Busch *et al*., 2012; Park *et al*., 2011).

The probable differences in results might be by use of different kinds of cells and methods (Windoffer *et al*., 2011).

Epithelial-mesencymal transition (EMT) is an important event that permit a polarized epithelial cell, to experience numerous biochemical conversions to deduce a mesenchymal phenotype of cell including increased migration, invasiveness, and significantly elevated resistance to apoptosis (Kalluri and Neilson, 2003).

Loss of keratin by phosphorylation is one of hallmarks in EMT (Kalluri and Weinberg, 2009). Therefore it is plausible that players implicated in perinuclear reorganization of keratin by phosphorylation are also involved in EMT. Accordingly, Tgase-2 involved in SPC or TPA-induced K8 phosphorylation and reorganization, is also implicated in TGF-β1-induced EMT (Park *et al*., 2013). ERK1/2, JNK and p38 are involved in phosphorylation of keratins and also TGF-β1-induced EMT (Park *et al*., 2013; Zhao *et al*., 2015). RKS2 involved in keratin phosphorylation, are involved in macrophage-stimulating protein-induced EMT (Ma *et al*., 2011).

Several phosphatases involved in dephosphorylation of keratins are also implicated in process of EMT. PRL-3 or PTP1B involved in keratin dephosphorylation also induced EMT (Wang *et al*., 2007a; Hiraga *et al*., 2013). In contrast, PP2A, DEDD, and AMPK reverses EMT (Lv *et al*., 2012; Bhardwaj *et al*., 2014; Chou *et al*., 2014; Kim *et al*., 2015). Therefore, several players in keratin phosphorylation seems have an important role in EMT and new target identification in keratin phosphorylation and reorganization might be new targets for controling EMT and metastasis.

New opportunity of compounds regulating the phosphorylation and reorganization of keratins

Modulation of keratin phosphorylation and reorganization is potential new way for controlling EMT and metastasis of cancer (Beil *et al*., 2003). Apparently, several kinase inhibitors including MAP kinase, might be used as agents for reducing phosphorylation and subsequent reorganization of keratins leading to EMT suppression. We attempted to identify compounds affecting the keratin phosphorylation and reorganization using SPC as inducer. We found that ethacrynic acid, a well-known diuretic, inhibits SPC-induced K8 phosphorylation, reorganization, and migration via Tgase-2 inhibition (Byun *et al*., 2013). We reported that BLT2 participates in the LTB4-induced K8 phosphorylation, reorganization and migration and LY255283 suppressed LTB4-induced phosphorylation and reorganization of keratins (Park *et al*., 2012). Therefore, BLT2 antagonists and Tgase-2 inhibitors might be new tools for controlling EMT and metastasis.

We also developed SPC blocker based on structure of SPC. Several compounds derived from SPC, suppressed SPC-induced K8 phosphorylation, reorganization and migration (Lee *et al*., 2014). We also screened microbial extracts and found that some microbial extracts suppress SPC-induced migration using SPC-induced migration of PANC-1 cells (Kang *et al*., 2011).

However, additional inducers released from tumor microenvironment that affect keratin phosphorylation and reorganization have not been identified. If several factors are released from tumor microenvironment and induced keratin phosphorylation and reorganization, blocking the common pathway would be an optimal strategy. Hence PP2A activator or inducers also might be good candidate for controlling keratin reorganization by dephosphorylating the phosphor serine residue of keratins or phosphorylated kinases (active forms) involved in phosphorylation of keratins.

CONCLUSION

Metastatic cancer cell is much softer than non-metastatic cancer cells (Cross *et al*., 2007). Viscoelasticity of cancer cells is related to keratin architecture. So elucidating new players to regulate the keratin phosphorylation and reorganization might provide new targets for suppressing the metastasis. Furthermore, novel compounds modulating the phosphorylation and reorganization of keratin might be a new hope for fighting against metastasis of cancer.

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