Connexin 43: Key roles in the skin (Review)

XIAO-FEI ZHANG and XIAOFENG CUI

Department of Biological Sciences and Biotechnology, School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Wuhan, Hubei 430070, P.R. China

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Abstract. Gap junctions are tightly packed intercellular channels that serve a common purpose of allowing the intercellular exchange of small metabolites, second messengers and electrical signals. Connexins (Cxs) are gap junction proteins. Currently, 20 and 21 members of Cxs have been characterized in mice and humans, respectively. Connexin 43 (Cx43) is the most ubiquitously expressed type of Cx in the skin. It is produced by various different types of skin cell, such as keratinocytes, fibroblasts, endothelial and basal cells, melanocytes and dermal papilla cells. At present, more evidence indicates that Cx43 has an important role in skin repair and skin tumor development, as well as in skin cell invasion and metastasis. In this review, current knowledge regarding the regulation and function of Cx43 is summarized and the therapeutic potential of regulating Cx43 activity is discussed.

Contents

1. Introduction

The skin forms a protective barrier between the internal organs and the environment by preventing invasion of pathogens, and fending off chemicals and physical assaults, as well as preventing the unregulated loss of water and solutes (1,2). Skin is arranged into three layers, including the epidermis, dermis and hypodermis (from top to bottom). Cutaneous blood vessels and nerve endings are presented in the dermis and hypodermis, while the epidermis is avascular with no neural tissue. Therefore, the cell-to-cell communications mediated by GJs provide a crucial mechanism for the integrity of the epidermal barrier and dermal supports (3).

GJ channels formed from Cxs are the predominant intracellular connections that regulate cell permeability and polarity. To date, 21 Cxs have been identified in humans and 20 Cxs in mice (4,5). Cxs are named according to their respective molecular weight, such as Cx26, Cx31, Cx43 and Cx57. The structure differences between them lie in the cytoplasmic loop and carboxyl (C) terminal region. The Cx subunit contains four hydrophobic transmembrane domains, comprising two extracellular loops, one cytoplasmic loop and one cytoplasmic N-terminal, as well as a C-terminal region. When functioning, six Cx subunits form a hemichannel in the plasma membrane that dock to another hemichannel in the plasma membrane of an adjacent cell to assemble a complete GJ channel (4). These channels allow the exchange and diffusion of various compounds up to a molecular mass of 1,000 Da, for example metabolites, ions, second messengers, water and electrical impulses (6,7). The half-life of Cxs is relatively short, ranging from 1.5 to 5 h in the majority of tissue and cell types (8,9). However, the mechanism of the balance between Cx synthesis and degradation remains elusive. Abnormal Cx expression has been reported to be associated with dysregulated cell proliferation, migration and wound healing rates (10).

There are 10 Cxs in human skin, including Cx43, Cx45, Cx40, Cx31, Cx26, Cx32, Cx30, Cx30.3, Cx41.8 and Cx39.3. Cxs display distinct function in the epidermis and dermis with overlapping expression (11). Among these 10 Cxs, Cx43 is the most ubiquitously expressed in the skin. It is produced by various different types of skin cell, such as keratinocytes, fibroblasts, endothelial and basal cells, melanocytes and dermal papilla cells (11,12).

2. Regulation of Cx43

Cx43 is encoded by gap junction α 1 gene (*GJA1*; MIMno. 121014). The normal expression (10), proper location (13) and accurate connection with other Cxs (14,15) are crucial for its function.

Cx43 expression is regulated at the transcriptional and post-transcriptional levels. One activator protein-1 (AP-1) and two Sp1 transcription factor (SP1) binding sites exist in the 5'-flanking promoter of Cx43 (16). Activated protein kinase C (PKC) and estrogen induce Cx43 transcription through AP-1 and SP1 sites (16,17). Wnt signaling and protease-activated

Correspondence to: Professor Xiaofeng Cui, Department of Biological Sciences and Biotechnology, School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, 122 Luoshi Road, Wuhan, Hubei 430070, P.R. China
E-mail: xfcui@whut.edu.cn

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receptor-1 (PAR-1) also increase the transcription level of Cx43 (18). Post-transcriptional regulation of Cx43 predominantly relies on its C-terminal region, which contains multiple phosphorylation sites and acts as the functional domain of Cx43. Tumor growth promoting factors, protein kinase and inflammatory mediators, such as PKC (19), mitogen activated protein kinase (20), Src kinase (21), casein kinase 1 (22) and PKA (23), may modulate phosphorylation of Cx43 through serine/tyrosine residues at the C-terminus and subsequently regulate subcellular localization of Cx43 and GJ formation. However, the effect of phosphorylation of Cx43 on GJ intracellular communication remains uncertain. The ubiquitin (24) and small ubiquitin-related modifier (25) system are important in post-transcriptional regulation of Cx43 GJs. In addition to post-transcriptional modifications, Cx43 interacts with a range of cytoskeleton proteins, including zonula occludens-1 (ZO-1), ZO-2, and α- and β-tubulin, to regulate cell adhesion and migration (26-28).

3. Implications of Cx43 in skin system

Increasing evidence indicates that Cx43 directly affects the proliferation and migration of keratinocytes and fibroblasts (29). Cx43 also participates in wound healing (30), hyperkeratosis (31) and tumor development of skin (32,33).

Cx43 in cutaneous wound healing. Normal skin regenerates after wounding or damaging. Wound healing is a complicated physiological process in which different types of cell, containing various growth factors and chemokines, are involved (34,35). There are four steps in the wound healing process: Hemostasis, inflammation, migration and proliferation, and remodeling (34,36). During wound healing, the expression of Cx43 varies and influences cell behaviors.

Numerous factors may regulate the expression levels of Cx43 during repair processes (Fig. 1). Subsequent to wounding, a high concentration of cyclic adenosine monophosphate at the wound site induces a reduction in Cx43 expression levels and other junction proteins at the plasma membrane, which subsequently destructs the cell junction and causes cytoskeleton remodeling (30). The activated AKT phosphorylates Cx43 at S373 and limits its interaction with ZO-1, potentially leading to activation and migration of keratinocytes in the skin (37).

The expression level of Cx43 changes dynamically during the wound healing process. On days 1 and 2 subsequent to injury, the mRNA and protein expression levels of Cx43 were significantly decreased at the wound edge, and readjusted to normal levels (like those in non-injured skin) from day 3 onwards in mice (10). By contrast, the level of Cx43 expression remains at a low level throughout the entire healing process in humans (29). Cx43 reduction has been shown to be associated with: i) Remodeling of the extracellular matrix (ECM) (38); ii) proliferation and migration of keratinocytes and fibroblasts (10,39,40); and iii) regulation of inflammatory responses through certain cytokines, chemokines or growth factors (38,40). Transient knockdown or artificial deficiency of Cx43 induces the proliferation and migration of keratinocytes and dermal fibroblasts, and enhances ECM production by upregulating collagen type I, collagen type III, matrix metalloproteinase-2 and transforming growth factor (TGF)-β1 (38). Additionally, Cx43 knockdown may decrease the expression levels of chemokine (C-C motif) ligand 2 and tumor necrosis factor (TNF)α, and elevate the expression levels of TGF-β1 and collagen α1, which affects the extravasations of neutrophils and macrophages involved in the wound healing process (40). Furthermore, the angiogenic potential of endothelial cells would be impaired following Cx43 knockdown (41,42). Although the role of Cx43 reduction in wound healing has been widely accepted, the underlying mechanism and signaling pathway involved in its function require further investigation to provide a solid theoretical basis for its clinical application.

Cx43 is crucial in chronic wound healing. Chronic wounds, such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers are an increasing issue worldwide (43). Diabetic ulcers, the most common diabetic complication, represent a major concern for patients and doctors with regard to quality of life and economics (44). Clinical and experimental evidence suggests that chronic wounds do not follow an orderly progression of wound healing (35). In the case of diabetic ulcers, abnormal expression of Cx43 was reported (45). As mentioned above, the Cx43 expression level is decreased at the wound borders during acute injury of normal human skin. By contrast, its expression level elevates by ~10-fold in human chronic diabetic foot ulcers at the wound edge (46). It was reported that the high glucose level of diabetic cells induces Cx43 expression, and subsequently represses filopodial extensions and fibroblast migration rates (46). However, Vinnik *et al* (47) observed a minor increase of Cx43 expression at the wound borders in patients with diabetes mellitus type II. In the study, the Cx43 expression levels at the wound edge increased by ~1.9 times following ozone therapy; however, the action mechanism of Cx43 in this case remains to be elucidated (47). A finding by Mendoza-Naranjo *et al* (48) that is consistent with the above-mentioned observations demonstrated an increased expression level of Cx43 in venous leg ulcers, and increased healing rates following Cx43 shRNA treatment (48).

The role of Cx43 is not simply to form GJ channels, but also to stabilize a series of proteins, such as N-cadherin and ZO-1, which are required for cell-to-cell adhesion and cell migration (45,46,48), which further illustrates that the sustained inhibition of Cx43 may be efficient for rapid or chronic wound healing.

Cx43 in keratoderma. Cx43 is tightly associated with keratinocyte behaviors. The human Cx43 gene, or *GJA1*, is located at human chromosome 6q22-q23 within the candidate region for the oculodentodigital dysplasia (ODDD) locus. A Cx43 mutation directly causes the pleiotropic phenotype of ODDD (49). It is proposed that the gene mutations (c.412G>A/p.Gly138Ser) (50) and deletions (dinucleotide deletion 780_781delTG) (51) causing truncation of the Cx43 C-terminus are necessary and sufficient for palmoplantar keratosis (PPK) development in ODDD patients. More recently, other mutations of Cx43 have been observed in various types of rare, inherited skin disorder characterized by keratoderma or hypokeratosis with other severe symptoms, including a heterozygous mutation (c.23G>T [p.Gly8Val]) of *GJA1* in a family with keratoderma-hypotrichosis-leukonychia totalis syndrome (31) and *de novo* missense mutations (A44V

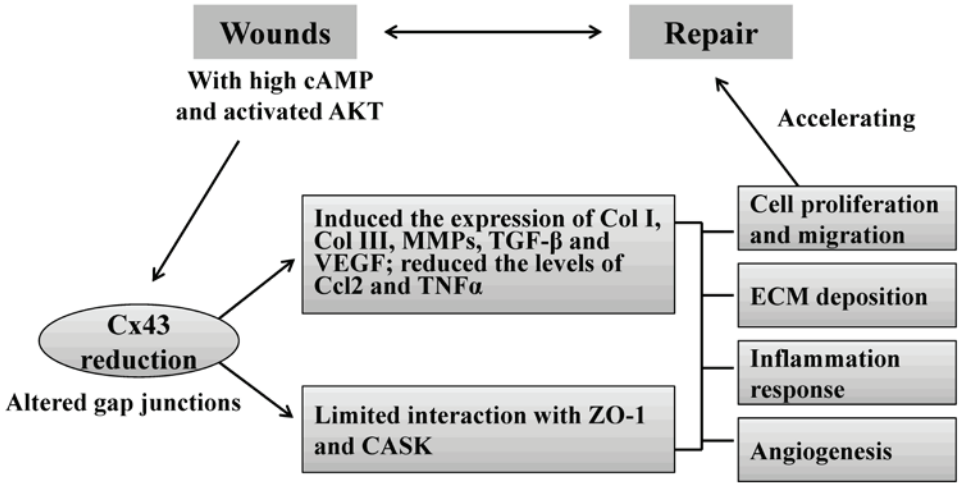

Figure 1. Schematic of the regulation and function of Cx43 in acute skin injury. Elevated cAMP and pAKT at wound edges alter the expression and phosphorylation levels of Cx43. By mediating the upregulation of Col I, Col III, MMP2, TGF-β1 and VEGF, and the downregulation of Ccl2 and TNFα, Cx43 participates in the important wound healing processes, including ECM remodeling, epidermal/dermal cell proliferation and migration, inflammation response and angiogenesis. In addition, dysphosphorylation of Cx43 affects its interaction with other proteins, such as CASK and ZO-1, and subsequently modulates cell permeability and migration. Overall, Cx43 integrates multiple pathogenic signals to regulate wound healing. Cx43, connexin 43; cAMP, cyclic adenosine monophosphate; pAKT, phosphorylated AKT; Col, collagen; MMP2, matrix metalloproteinase-2; TGF-1, transforming growth factor; VEGF, vascular endothelial growth factor; Ccl2, chemokine (C-C motif) ligand 2; TNFα, tumor necrosis factor; ECM, extracellular matrix; CASK, calcium/calmodulin-dependent serine protein kinase; ZO-1, zonula occludens-1.

and E227D) of *GJA1* in erythrokeratodermia variabilis et progressiva (52). Mechanistically, *GJA1* mutations lead to disruption of Cx43 membrane localization and aggregation in the Golgi, resulting in excessive opening of hemichannels and cytoplasmic Ca^{2+} overload, and subsequent keratinocyte apoptosis and hyperkeratosis.

In addition to the above-mentioned mutations, Cx43 participates in epidermal keratinization by interacting with other members of the Cx family. For example, Cx26 mutations, G12R/N14Y or H73R/S183F, directly caused keratitis-ichthyosis-deafness and PPK syndrome, respectively. During these syndromes, Cx26 mutants may interact with Cx43 more efficiently and exacerbate Cx43 hemichannel activity, thus increasing cell membrane permeability and resulting in ATP release and Ca^{2+} overload (14,15). These studies further demonstrate the important role of Cx43 in genetic skin disorders.

Cx43 in melanoma and non-melanoma skin cancer. Cx43 is closely associated with tumor initiation and development. In skin cancer, Cx43 is overexpressed in malignant melanomas when compared with the normal and benign nevi (32,33). The upregulation of Cx43 was associated with an enhanced cell adhesion and invasion of malignant tumor cells (53,54). By acting as a downstream effecter of PAR-1, Cx43 also mediates melanoma metastasis and intracellular communication between the tumor microenvironment and the metastatic tumor cells (46). The roles of Cx43 in melanoma implicate it as an oncogene; however, various independent groups obtained opposing results. Schiffner *et al* (55) identified Cx43 as a downstream target of nuclear RNA-binding protein $p54^{nrb}$. Cx43 knockdown mediated by $p54^{nrb}$ promotes cell proliferation and migration in human melanoma cell lines. In mouse melanoma cell lines, Cx43 reduction induced the expression of vascular endothelial growth factor and tumor angiogenesis (56). Similarly, in human melanoma cell lines, Cx43 overexpression reduced melanoma growth and metastasis, and increased TNFα-induced cell apoptosis (57). These controversial results may be due to differences between cell lines, experimental conditions and test points in the studies.

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the major subtypes of non-melanoma skin cancer (58). Stelkovics *et al* (59) observed a higher expression level of Cx43 in BCC than that in SCC, indicating that Cx43 prevented metastatic invasion of BCC (59). In a previous study, immunofluorescence and immunoelectron microscopy were used, and the expression level of Cx43 was relatively low in the basal layer of human normal skin; but Cx43 was not detectable in BCC or SCC, which consequently led to a small number of GJs in BCC and SCC (60). However, the underlying mechanisms of Cx43 function in melanoma and non-melanoma skin cancers remain poorly understood and require further study and discussion.

Cx43 in skin development. Cx43 contributes to epidermal and follicular morphogenesis. During human fetal epidermal development, Cx43 is expressed at the later stages (88 days) of estimated gestational age (61). In rat and mouse skin, Cx43 is expressed in all of the epidermal layers in the early phase of development (62,63), and is detected in hair follicles and the arrector pili muscles (12,64). The role and underlying mechanism of Cx43 in epidermal development remains largely unknown. During mouse ovarian folliculogenesis, Cx43-mediated GJs are required for coupling between granulose cells and continued follicular growth (65). In addition, Cx43 evolves in skin cell differentiation. Dyce *et al* (66) compared stem cells from the skin of wild-type and Cx43 knockout newborn mice. The authors found reduced cell migration rates and decreased expression levels of the pluripotency markers, octamer-binding transcription factor 4 and Nanog in Cx43-deficient stem cells, indicating the role of Cx43

Table I. Cx43 blockers and their functions in the cutaneous system.

Author, year	Cx43 blockers	Type	Topological position	Oligos/peptide sequence	Specificity	Function	(Refs.)
Mori *et al*, 2006; Wang *et al*, 2007; Qiu *et al*, 2003	Oligodeoxy-ribonucleotides	Oligodeoxy-ribonucleotides		GTAATTGCGG CAGGAGGAAT TGTTTCTGTC	Specific	Accelerates wound healing with reduced scar formation in normal mouse skin and diabetic rat skin	(40,45,70)
Boitano *et al*, 1998	Gap15	Peptide	Intracellular loop	EIKKFKYGIEEHC	Specific	N.R.	(71)
Oviedo-Orta *et al*, 2000	Gap18	Peptide	Intracellular N-terminal	MGDWSALGK LLDKVQAC	Specific	N.R.	(72)
Wang *et al*, 2013	Gap19	Peptide	Intracellular domain	KQIEIKKFK	Specific	N.R.	(73)
	Gap26	Peptide	Extracellular loop 1	VCYDKSFPISHVR	Non-specific	Increases migration rates of keratinocytes and fibroblasts	
	Gap26M	Peptide	Extracellular loop 1	VCYDKSFPISHVR	Non-specific	Increases migration rates of keratinocytes and fibroblasts	
	Gap27	Peptide	Extracellular loop 2	SRPTEKTIFII	Specific	Enhances wound closure by inducing primary mouse keratinocytes migration to wound edges	
Oviedo-Orta *et al*, 2000	Gap35	Peptide	Extracellular loop 1	AWGDEQSAFR CNTQQPGC	Specific	N.R.	(72)
	Gap36	Peptide	Extracellular loop 2	KRDPCPHQV DCFLSRPTEK	Specific	N.R.	
Ongstad *et al*, 2013	αCT1	Peptide	C terminus	N.R.	Specific	Enhances wound healing in animal models of skin and heart	(76)
Goldberg *et al*, 1996 Davidson and Baumgarten, 1988	Octanol and CBX	Chemicals	N.R.	N.R.	Non-specific	Blocks gap junction communication in human fibroblasts at low concentration	(78,79)

N.R., not reported in cutaneous system.

in maintaining the multipotency of skin stem cells (66). The results are partially consistent with a previous study, in which the expression level of Cx43 was maintained at a higher level in an undifferentiated epidermis and markedly decreased in the differentiation of the epidermis (12).

Therapeutic modalities based on regulating Cx43 activity. Increasing evidence has identified a positive correlation between Cx43 inhibition and wound healing rates (67,68). Furthermore, Cx43 reduction relieves inflammation in chronic wounds (69). Currently, drug design and development based on Cx43 is an important research area. There are two methods for artificially downregulating Cx43, which involve antisense oligodeoxyribonucleotides (ODNs) and mimetic peptides (Table I). Antisense ODNs may have accelerated wound healing and reduced scar formation in normal mouse skin and diabetic rat skin (40,45,70). Cx43 mimetic peptides include Gap 15 (71), Gap 18 (72), Gap 19 (73), Gap 26 (74), Gap 26M (Gap26 modified with acylation to improve solubility and stability) (74), Gap 27 (74), Gap 35 (72) and Gap36 (72). Among these, the most evaluated peptides in skin cells are Gap26, Gap26M and Gap27. Gap26 and Gap26M directly interacted with amino acids 63-75 of the extracellular loop 1 of Cx43 (VCYDKSFPISHVR); Gap27 mimicked amino acids 204-214 on the extracellular loop 2 of Cx43 (SRPTEKTIFII) (74). Gap26 and Gap26M are non-Cx43 specific, whereas Gap27 is Cx43-selective (74). Various studies have demonstrated that peptide treatment significantly increases the migration rates of keratinocytes and fibroblasts to wound edges (74,75). In addition to the above-mentioned peptides, Ongstad *et al* (76) designed a cell-permanent α-connexin carboxyl-terminal (αCT1) peptide based on the C-terminus of Cx43. The peptides encapsulated in pluronic F127 thermogel and methylcellulose demonstrated a significant accelerating effect on wound healing in pre-clinical animal models of the skin and heart. An external application of αCT1 on wound healing is now set to proceed into phase I and II clinical trials (76). Gap19 is currently applied for the treatment of myocardial ischemia/reperfusion injury and, to the best of our knowledge, there are no reports regarding its application in wound healing (73). Other peptides, including Gap15, Gap18, Gap35 and Gap36 were reported as Cx43 selective blockers; however, the function of these in cutaneous systems remain elusive (71,72). Various chemicals, including octanol and 18β-glycyrrhetinic acid and its water-soluble derivative, carbenoxolone, block intercellular junctional communication by targeting Cxs (77-79).

Notably, primary diabetic cells exhibited less susceptibility to Cx43 inhibitors (80,81); therefore, the clinical application of administering Cx43 peptides for the treatment of chronic diabetic wounds requires further critical consideration. As the mode-of-action of Cxs is not unique, selective downregulation of one Cx rather than using broad-spectrum inhibitors would be preferable. Furthermore, the functional mechanism and effects of Cx43 mimetic peptides on other tissues have yet to be determined.

4. Conclusion

Recent studies on Cx43 in the skin clearly demonstrate that Cx43 is important in human skin biology and pathology. The expression level and activity of Cx43 are strictly regulated in certain situations. Downregulation of Cx43 following tissue injury significantly reduces ECM deposition, inflammation response and scar formation, and accelerates wound healing rates. Currently, Cx43 expression levels and functions have been presented in skin injury, skeletal muscle regeneration (82), ischemia/reperfusion injury (83) and cornea repair (84). The Cx-associated compounds are in development and indicate promising therapeutic opportunities in preclinical evaluation (85). Non-toxic Cx43 specific inhibitors may also be effective for the treatment of wounds, skin cancer or other skin associated disorders. Therefore, it is considered urgent to investigate the underlying mechanisms and clinical potential of Cx43 in the skin.

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