# Review of the molecular mechanisms in wound healing: new therapeutic targets?

**Abstract:** The restoration of the skin barrier in acute and chronic wounds is controlled by several molecular mechanisms that synergistically regulate cell kinetics, enzymatic functions, and neurovascular activation. These pathways include genetic and epigenetic activation, which modulate physiological wound healing. Our review describes the genetic background of skin repair, namely transcription-independent diffusible damage signals, individual variability, epigenetic mechanism, controlled qualitative traits, post-translational mechanisms, antioxidants, nutrients, DNA modifications, bacteria activation, mitochondrial activity, and oxidative stress. The DNA background modulating skin restoration could be used to plan new diagnostics and therapeutics.

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Wound healing may be considered the result of sequential steps, progressing gradually to full skin restoration.[1] The regulation of gene expression in wound healing, involving cell kinetics, enzymatic functions, and neurovascular activation, among other processes, is quite puzzling.[2] Furthermore, it shares some gene expression patterns with the process of invasive tumour development.[3] Wound healing is phylogenetically carefully preserved,[1] in fact, *in vivo* studies from invertebrate and vertebrates displayed similar gene expression patterns between wound healing and developmental processes.[2]

## Molecular mechanisms regulating wound healing

### Transcription-independent diffusible damage signals

At the beginning of wound healing, some specific transcription-independent diffusible damage signals have been described, in both vertibrate and invertebrate models.[2] These include $Ca^{2+}$ waves, hydrogen peroxide ($H_2O_2$) gradients, and the cellular release of adenosine 5′ triphosphate (ATP).[2] Generally speaking, the injury quickly increases the intracellular $Ca^{2+}$ concentration, which is known to modify gene transcription through protein kinase C (PKC), $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK),[4] and reactive oxygen species (ROS), such as $H_2O_2$.

ROS are simultaneously dangerous and precious molecules. When present at high intracellular concentrations, ROS can cause substantial damage to crucial biological processes through oxidative stress. However, when present at very low levels, ROS are extremely effective signalling molecules. By oxidizing thiol groups on Cys residues, ROS changes protein reactivity towards downstream targets. ROS can also alter protein phosphorylation levels and cause other post-translational modifications. In the context of wound healing, ROS signalling is involved in cell attraction, migration, adhesion, and immune cell activation.[5] The ROS, $H_2O_2$, interferes with haemostasis, inflammation, angiogenesis and re-epithelialisation. Although the direct targets of $H_2O_2$ molecules, during these steps, have only been partially characterised, it is known that $H_2O_2$ generated by the electron transfer mechanism, is a rapid signal of injured tissues,[6] triggering chemotactic signals, and alerting the immune system, both *in vitro* and *in vivo*.[2]

The release of ATP, and its activation of purinergic receptors, affects the wound healing process.[7] In normal conditions, intracellular concentrations of ATP are very high (≈100mM), whereas extracellular concentrations are considerably lower (≈10nM), and therefore ATP release is favoured. *In vitro* studies of human corneal and bronchial epithelia show that mechanical injury causes rapid and high levels of ATP release from the damaged cells into the extracellular space.[8] Furthermore, the extracellular ATP, which leads to further autocrine ATP release (putatively through P2 purinergic receptors),[9] is recognised by P2Y receptors on microglia and the surrounding tissue. This triggers a dynamic immune response at the border of the injured and undamaged tissue.

The DNA damage in epithelial cells is recognised by P2Y receptors on adjacent healthy cells, which relay cytoplasmic signals involving intracellular $Ca^{2+}$ and metalloproteinase (MMPs) activation. This results in

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the release of specific growth factors, such as epidermal growth factor (EGF), activating a wound healing cascade.

Actomyosin structures are important in early wound healing.[2] Tissue injury stimulates rapid $Ca^{2+}$ waves that activate RHO GTPases, and promote actin polymerisation and actomyosin contractility to maintain stromal integrity. Furthermore, $Ca^{2+}$ can directly activate actin-severing proteins, such as calpain and gelsolin, leading to increased actin dynamics.[1] $Ca^{2+}$ can also potentiate c-Jun N-terminal kinases (JNK) and mitogen-activated protein kinase (MAPK) signalling, which induces the transcription factor activation and increases expression of wound response genes, including several cytoskeletal regulators.[2]

A mechanism for the activation of damage signals is mechanic-sensing and mechanic-transduction. Cytoplasmic barriers are the first protection against damage. The surface tensioactivity is a shelter preserving intracellular content, but modifications of proteins of protein conformation can activate an alarm system. For example, ion channels react to membrane pressure by changing their permeability.[10] The efflux or internalisation of ions, such as $Ca^{2+}$, could therefore be facilitated when membranes undergo tension changes following injury. Mechanic-sensory $Ca^{2+}$ channels, such as transient receptor potential (TRP) channels, have been implicated in damage signaling.[11] Therefore, damage to cell membranes could trigger the formation of $Ca^{2+}$ waves by inducing the opening of TRP channels and enhancing sudden $Ca^{2+}$ influx.[12] The resulting high levels of intracellular $Ca^{2+}$ may regulate ROS activity, which would lead to increased formation of $H_2O_2$.[13] Furthermore, sudden increases in intracellular $Ca^{2+}$ may promote ATP gradient formation (Table 1).[11]

### Gene expression and individual variability

Gene expression is one of the early cellular responses to wound healing. During wound repair marked changes in gene expression are induced.[14] Response to tissue injury involves multiple cellular and extracellular events,[14] including coagulation, inflammation, re-epithelialisation, and angiogenesis. These are followed by fibroplasia with collagen synthesis, wound contraction and, finally, tissue remodelling. These cellular and extracellular events require the activation, or silencing, of many genes, to coordinate the response of the different cell types involved in healing. A key issue in understanding the molecular mechanisms of wound healing is to identify differentially expressed genes, and associated signaling cascades that are preferentially regulated in a development-, age-, tissue/cell type-, and time-dependent manner during the early events of the wound healing.

Our focus is the expression of specific genes following tissue injury, which are individually tailored and can lead to 'restitutio ad integrum' or hyperthrophic/keloid scar.[15] These include the gene expression of extracellular molecules, such as collagen and proteases, as well as the molecules involved in cell-cell signalling, such as growth factors.[16] However, differences in the expression of isolated functional genes alone may not sufficiently explain clinical variations of wound healing.

A methodologic strategy may be to identify mRNA differential display profiling from isolated genes, differently expressed during wound healing in *in vivo* models.[17] From genomic analysis of physiological and pathological conditions, DNA sequence data on the whole healing process have been developed.[14] Using complementary DNA (cDNA) technology, it is possible to quickly analyse 4000 genes of wound specimens collected from different body areas, in order to reveal gene expression patterns (Table 1).[14]

### Epigenetic mechanisms

Epigenetic mechanisms are involved in the wound healing process. Although not fully understood, they are based on molecular chromatin modifications, which consequently influence protein expression. The nucleosome is the elementary unit from which chromatin is comprised, and consists of eight histone proteins (an octamer) and 146 base pairs of DNA. This octamer is based on the proteins H3 and H4, organised as a tetramer, and H2A and H2B, organised as dimers. The chromatin composition depends on post-translational modifications to histones. Specifically, the open chromatin conformation in the DNA is accessible to many transcription factors, allowing the gene transcription, while the closed chromatin conformation does not allow transcription.

The epigenetic mechanisms regulating chromatin structure and histones modifications consist of methylation, phosphorylation, ubiquitination and acetylation.[18,19] Acetylation is performed by the activity of histones acetyltransferases (HATs) and histone deacetylases (HDACs) enzymes. Hyperacetylation of lysine residues at the ε-amino group in the N-terminal of histones by HATs enzymes results in increased gene transcription; while deacetylation by HDACs enzymes is associated with reduced gene transcription.[18] DNA methylation, which results in gene silencing, occurs at the cytosine base located in CpG islands, which are regions of the genome containing CpG dinucleotides. Another epigenetic mechanism involves regulation by microRNAs, single stranded RNAs which are not translated into protein.[20] Their role is in binding complementary regions of mRNA blocking gene translation. Previous studies revealed that up to 3% of the genome encodes for microRNAs.[21] Several studies highlighted the role of epigenetic mechanisms in regulating wound healing, although the knowledge of the molecular mechanism is limited.[22] For example, Taganov et al.[23] described 200 microRNAs expressed in human monocytes, which were activated by various pro-inflammatory cytokines and microbial endotoxins. An example is the microRNA (miR)-146, which was found to be induced by transcription factor nuclear factor κB (NFκB), and proposed to regulate innate immune responses, such as cytokines and Toll-like

**Table 1. Molecular mechanisms regulating the wound healing process**

| Molecule/event | Activity | Molecular mechanism | Role in wound healing |
|---|---|---|---|
| $Ca^{2+}$ | Transcription-independent diffusible damage signals | Tissue injury leads to a rapid increase in intracellular $Ca^{2+}$, which is known to modify gene transcription through protein kinase C and $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) | Promotion of actin polymerization and actomyosin contractility of fibroblast and keratocytes<br>Increased actin dynamics<br>Increased expression of wound response genes |
| $H_2O_2$ | Transcription-independent diffusible damage signals | Implicated in establishing chemotactic signals that alert the immune system to damage | Modulation of hemostasis, inflammation, proliferation, angiogenesis, epithelialisation and remodelling steps of wound healing |
| ATP | Transcription-independent diffusible damage signals | Mechanical injury causes a rapid and considerable ATP release by damaged cells into the extracellular space | Activation of the wound healing cascade |
| miR-146 | MicroRNA | Activation – epigenetic signal | Activation of the NFκB<br>Regulation of innate immune responses |
| miR-125b | MicroRNA | Inhibition – epigenetic signal | Inhibition of TNFα<br>Regulation of inflammatory genes |
| miR-221 and miR-222 | MicroRNA | Activation – epigenetic signal | New vessel formation |
| miR-146a | MicroRNA | Activation – epigenetic signal | Production of ECM proteins in chronic diabetes complications |
| miR-27b | MicroRNA | Activation – epigenetic signal | Activation of cell proliferation and adhesion<br>Inhibition of oxidative stress responses<br>Improvement of new vessel formation |
| miR-210 | MicroRNA | Activation – epigenetic signal | Inhibition/activation of keratinocytes proliferation |
| miR-203 | MicroRNA | Activation – epigenetic signal | Activation of keratinocytes proliferation |
| Metabolic memory | DNA methylation | Epigenetic signal | Diabetic foot fibroblasts and diabetic foot ulcer fibroblasts had lower global DNA methylation compared with non-diabetic foot fibroblasts |
| Polycomb Group (PcG) class of genes | Chromatin gene repression | Epigenetic signal | Down regulation of three repressive PcG proteins (Eed, Ezh2, and Suz12) during wound healing |
| Trithorax Group (trxG) class of genes | Chromatin gene activation | Epigenetic signal | Upregulation of two activating trxG members (Jmjd3 and Utx) during wound healing |
| Quantitative trait loci (QTL) | Controlled qualitative trait | Individual variability of the gene expression | Gene expression variance influences the rate and time of wound healing efficacy |
| Fibronectin (pFN and cFN) | Gene polimorfisms | Alternative splicing | The splicing depends on the cell type, the cell function and the stage of development |
| Poly (ADP-ribose) polymerase (PARP) enzymes | PARylation | PARPs cut off nicotinamide from NAD+ and attach the remaining ADP-ribose units to suitable protein acceptors. DNA damages active PARP enzymes | Acceleration of wound closure. Acceleration of keratinocytes migration. Stimulation of the synthesis of inflammatory mediators and the wound repair activity of keratinocytes |
| MtROS | Mitochondrial Reactive Oxygen Species | Production of ROS in mitochondria | Promotion of actin-based healing of epithelial wounds<br>Anti-bacterial activity<br>Regulation of endothelial cells migration |
| SkQ1 and SkQR1 | Mitochondria-targeted plastoquinones | | Myofibroblasts synthesis<br>Acceleration of the resolution of the inflammatory phase, formation of the granulation tissue, new vessel formation and re epithelialisation. Augmentation of the amount of myofibroblasts involved in the deposition of ECM proteins and growth factors. Stimulation of fibroblasts to synthesise TGF–β, which targets the motility of endothelial cells *in vitro*, and promotion of angiogenesis |

receptor signalling in monocytes, by negative feedback regulation of tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1).

Diabetes-associated non-healing wounds in mice are improved by mesenchymal stem cells, at least in part, mediated by an increase in miR-146a expression, which represses pro-inflammatory genes within the wound. [24] Tili et al.[25] identified the miR-125b transcription, which was blocked by NFκB and has been shown to repress TNFα, a key pro-inflammatory cytokine. Villeneuve et al.[26] demonstrated that miR-125b epigenetically regulates inflammatory genes in cultured vascular smooth muscle cells from type 2 diabetic db/db mice through a mechanism involving downregulation of the histone H3 lysine-9 methyltransferase Suv39h1. The role of microRNA was also investigated in angiogenesis. In human umbilical vascular endothelial cells (HUVEC), miR-221 and miR-222 played angiogenic effects by blocking the translation of c-Kit, a receptor for the pro-angiogenic ligand stem cell factor (SCF).[26]

miR-146a has been involved in extracellular matrix (ECM) protein production in an *in vitro* model (endothelial cells from large vessels and retinal microvessels) at different glucose concentrations. Increased production of ECM proteins, such as fibronectin, is a characteristic feature of all chronic diabetes complications. Fibronectin transcripts are upregulated because of abnormal signalling mechanisms in hyperglycaemia.[27] Wang et al.[28] demonstrated that the miR-27b rescues impaired bone marrow–derived angiogenic cell (BMAC) function *in vitro* and *in vivo* in type 2 diabetic mice. Results showed that the miR-27b expression and BMAC function was reduced in diabetic mice, the addition of miR-27b mimic improved many BMAC functions, including proliferation, adhesion, tube formation, and delayed apoptosis, but it did not reverse the effecs on migration.[28] Furthermore, on miR-27b mimic transfection, elevated thrombospondin-1 expression was reduced in the BMACs of diabetic mice. While the inhibition of miR-27b in BMACs reduced angiogenesis, this was reversed by thrombospondin-1 small interfering RNA (siRNA).[28] Moreover, the addition of miR-27b suppressed the pro-oxidant protein p66shc and mitochondrial oxidative stress, contributing to its protection of BMACs function. miR-27b also suppressed semaphorin 6A to improve BMACs function in diabetic mice (db/db mice). Using the luciferase binding assay, it has been suggested that miR-27b directly targeted thrombospondins, p66 shc, and semaphorin 6A.[28] Finally, miR-27b improved skin wound closure of diabetic mice BMACs ,with a concomitant augmentation of wound perfusion and capillary formation. These findings suggest that miR-27b rescues impaired BMACs stimulated angiogenesis due to thrombospondin suppression, semaphorin 6A expression, and p66shc-dependent mitochondrial oxidative stress, and improves BMAC therapy in wound healing in type 2 diabetic mice.[28]

Keratinocyte-dependent functions also target microRNAs. For example, miR-210 has been associated with repressing keratinocyte proliferation,[29] targetting transcription factor E2F3, a key promoter of keratinocyte proliferation.[29] miR-203 downregulates the transcription factor p63 in primary keratinocyte cells *in vitro*.[30] Based on these studies, epigenetic-based medicines may have a role as new therapies to aid wound healing.

Scientific evidence has demonstrated that there is a central mechanism involved in the metabolic memory of the hyperglycemia-associated epigenetic patterns.[31] It was established that heritable transmission of epigenetic patterns may be responsible for the persistent hyperglycemia, despite removal of the glycemic insult.[32,33] This metabolic memory concerns DNA methylation and microRNA expression patterns.[31] It is evident that hyperglycemia is responsible for diabetes-related consequences, such as chronic wounds. Park et al.[31] pooled a cohort of patient-derived cell lines from diabetic foot ulcer (DFU) fibroblasts (DFUF), and site- and age-matched diabetic foot fibroblasts (DFF) and non-diabetic foot fibroblasts (NFF). The goal was to investigate global and genome-wide DNA methylation patterns by chromatography/mass spectrometry. Results showed that DFFs and DFUFs had lower global DNA methylation compared with NFFs ($p<0.03$). Moreover, the authors identified sustained DNA methylation patterns in patient-derived fibroblasts, from patients with diabetes, after prolonged passage in normal glycemic conditions.[31] This suggests that there is a metabolic memory regulated by epigenetic mechanisms, which may influence wound healing processes, and may be a target for therapeutic strategies.

Chromatin can be modified by a vast number of highly conserved proteins, which are involved in designating transcriptionally active or silent regions.[34] This suggests that chromatin modifications, by proteins, may play a role in processes where the gene expression is altered, such as wound healing. The polycomb group (PcG) class of genes is involved in chromatin gene repression; while trithorax Group (trxG) class of genes is associated with chromatin gene activation.[35] Shaw and Martin[36] showed, in a murine model of wound healing, the downregulation of three repressive PcG proteins (Eed, Ezh2, and Suz12), and the upregulation of two activating trxG members (Jmjd3 and Utx) (Table 1).

### Controlled qualitative traits

The wound healing process may also be considered as a controlled qualitative trait, which differs between individuals.[37] A previous study[38] demonstrated that the MRL/MpJ-Faslpr (MRL-F) strain of mice could completely heal an ear-punched hole (2mm diameter) within 30 days, with no scar tissue. In contrast, the C57BL/6 strain of mice healed only 40% and SJL/J

<25% of the original hole, with scar tissue, at the same time point. The rapid wound healing in MRL-F mice is a genetically controlled quantitative trait, according to McBrearty et al.[39] The authors used MRL-F and C57BL/6, F2 intercross at one time point, which resulted in the identification of five qualitative trait loci (QTL) explaining unknown percentage of variance in F2 mice. The five QTL can contribute to the healing phenotype in two different types of genetic crosses, increasing variability. The MRL mouse is thus an ideal model to define the molecular mechanisms of wound-repair/regeneration in mammals.[37] To further identify genetic mechanisms controlling wound healing, Masinde et al.[37] used F2 population from progenitor strains of different genetic origin (MRL/MpJ and SJL/J). The objectives of this study were to map fast-healer genes using two genetically extreme progenitor strains (MRL/MpJ and SJL/J) and to identify epistatic interactions at multiple time points. Results showed the identification of the same QTLs at each time point that explained 70% of the variance in F2 mice and that QTL together with epistatic interactions could promote wound healing (Table 1).

### Antioxidants

Antioxidants are key molecules in regulating wound healing. Among these, 3,5,4 0–trihydroxystilbene, a polyphenolic phytoalexins found in green vegetables, citrus fruits and red grape wine, induced the synthesis of vascular endothelial growth factor (VEGF), stimulating the differentiation of endothelial cells from bone stem cells.[40] San Miguel et al.[41] demonstrated that concentrations (0.1–1mM) of bioactive pure polyphenol and turmeric derivative mixtures, such as resveratrol (R), ferulic acid (F), phloretin (P) and tetrahydrocurcuminoids (T) had *in vitro* beneficial effects on human oral fibroblast (obtained from human gingival and periodontal tissues) migration and proliferation after 72 and 96 hours. However, the mixture of phloretin, ferulic acid and resveratrol (PFR; 1mM and 0.1mM) and the mixture of phloretin, ferulic acid and tetrahydrocurcuminoids (PFT; 1mM) significantly increased DNA synthesis in human oral fibroblasts obtained from periodontal tissues after 48 hours. These results suggest that specific concentrations of this bioactive antioxidant compound may have beneficial effects on functional mechanisms regulating fibroblast migration and proliferation during gingival healing or periodontal repair.[41]

The antioxidant T, extracted from the roots of *Curcuma longa*, is effective in wound healing, since it stimulates the synthesis of TGF-β1 and iNOS during the proliferative step.[42] Curcumin (diferuloylmethane), a bioactive constituent from *Curcuma longa,* has remarkable anti-inflammatory, antioxidant, and anticarcinogenic activity.[43] *In vitro* and *in vivo* studies demonstrated that curcumin has been effective in the inhibition of the expression of different inflammatory cytokines such as TNFα, IL-1, and IL-8.[44] Curcumin is a potential scavenger of oxidised free radicals, and it increases the level of glutathione during apoptosis.[45] Evidence shows that curcumin regulates expression and activity of MMPs involved in wound healing. Swarnakar et al.[43] showed that curcumin downregulated MMP-9 activity and upregulated MMP-2 activity in mice with an indomethacin-induced gastric ulcer. An oral dose of curcumin (60mg/kg) reduced the gastric damage caused by indomethacin by 85%. Curcumin was also effective at accelerating the healing of gastric ulcers by a MMP-dependent process. In fact, in the gastric ulcerative mucosa of mice, wound healing progression positively correlated with reduction of activity of MMP-9 and with augmentation of MMP-2 activity.[43] It is important to remember that MMP-9 and MMP-2 regulated the turnover of matrix proteins because together they are capable of degrading basement membrane proteins like gelatin, collagen IV, collagen V, elastin, and fibronectin.[43] Furthermore, MMP-2 is constitutively expressed in many cell cultures; while MMP-9 expression is induced by pro-inflammatory cytokines, growth factors, and cell/stroma interactions.[46]

Coenzyme $Q_{10}$ (Ubiquinone $Q_{10}$, $CoQ_{10}$), a vitamin-like benzoquinone compound, has been shown to aid corneal wound healing.[47] Acting to deliver energy through mitochondrial apoptosis, it modulates free radical scavenger activity and increases respiratory rate.[48] The $CoQ_{10}$ modulates the permeability transition pore (PTP), a mitochondrial inner membrane conductance channel, being a potential mitochondrial inhibitor of apoptotic signal transduction.[49] Mencucci et al.[47] evaluated the potential protective effects of $CoQ_{10}$ at different concentrations, on human corneal cells (HCE) where the mitochondrial activity and survival were evaluated by means of 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyl-tetrazolium (MTT) reduction, and lactic dehydrogenase (LDH) release. Oxygen consumption and mitochondrial membrane potential were also evaluated. The effect of two $CoQ_{10}$ drops of ultraviolet B (UVB) exposure-(312nM) induced conjunctival vessel hyperaemia and corneal recovery after ethanol-induced corneal lesion was examined in *in vivo* rabbit models. The results showed that in UVB-exposed HCE cells, $CoQ_{10}$ addition led to increased survival rate and mitochondrial function. Oxygen consumption was maintained at control levels and ATP levels remained normal in the $CoQ_{10}$-treated cells. Interestingly, in the *in vivo* model, there was a $CoQ_{10}$ dose-dependent reduction in UVB-induced vessel hyperaemia. Finally, in the model of corneal epithelium removal, 12 hours after surgery, animals treated with $CoQ_{10}$ showed a reduction of damaged area compared with the vehicle controls, which lasted for 48 hours. These data suggest that $CoQ_{10}$ reduces corneal damages after UVB exposure *in vivo* and *in vitro* by preserving mitochondrial function. In addition, the administration of $CoQ_{10}$ after corneal epithelium removal, promoted corneal wound healing.

Alleva et al.[50] evaluated the effects of 300mg α-lipoic acid (LA, one capsule) in 20 patients affected by chronic wounds, undergoing hyperbaric oxygen therapy (HBOT). The protocol consisted of the patient taking one capsule, one hour before oxygen exposure, and one capsule immediately after the therapy for 30 consecutive HBOT treatments (one session/day). LA supplementation efficiently reduced both the lipid and DNA oxidation induced by oxygen exposure in the treated group, compared with the placebo group ($p<0.05$). LA exerted its antioxidant activity by directly interacting with free radicals or by recycling vitamin E. An inhibitory effect of LA on the pro-inflammatory cytokine IL-6 was also observed.[50]

It has been suggested that resveratrol possesses inhibitory activity on MMPs. In fact, sirtuin-1 (SIRT1) is an MMP specifically regulated by resveratrol. Being an agonist of SIRT1, resveratrol inhibits the transcription of MMPs in the skin.[51] Specifically, MMP-8 and MMP-9 play a key role in diabetic wound healing since these enzymes cause degradation of collagen and other structural constituents of skin ECM.[52] Thus, high levels of MMP-8 and MMP-9 in the bed of diabetic ulcers are predictors of poor wound healing.[53] On the contrary, resveratrol supplementation may promote healing of ulcers in patients with diabetes.

### Post-translational mechanisms

Fibrin deposition plays a key role in wounds, since it prevents the healing.[54] Chronic leg ulcers are characterised by the presence of dermal 'fibrin cuffs' which are composed of fibrin, laminin, fibronectin, tenascin, collagen and trapped leucocytes, enabling the exchange of gases, growth factors and nutrients between plasma and dermis, and leading to tissue anoxia, ulceration, and inhibition of angiogenesis.[55] Proteolytic lysis of fibronectin releases fragments, which have been shown to induce cell proliferation[56] and migration.[57] Evidence exists suggesting an alternative pre-mRNA splicing mechanism and fibronectin are involved in wound healing. Fibronectin is a multiple isoform molecular complex, including the forms plasma FN p(FN) and cellular FN filaments (cFN, a dimeric or multimeric form at the cell surface and in ECM).[58] Fibronectin protein isoforms depend on alternative pre-mRNA splicing mechanism. Specifically, both isoforms of fibronectin are encoded by a single large gene, Fn1 (50 kb, 50 exons),[59] three regions undergo alternative splicing, extra domain A (EDA, EIIIA), extra domain B (EDB, EIIIB) and a variable region (V, IIICS), depending on the cell type, the cell function and the stage of development (Table 1).[58] In the human, about 20 different mRNA-encoding protein subunits are produced.[60] The full-length nature of some variants has not been determined. Alternative splicing presumably allows a cell to produce the type of FN that is most suitable for the needs of certain tissue or cellular functions.[61]

Proteins containing arginine/serine-rich (RS) domains play a relevant role in the regulation of synthesis and modification of pre-mRNA.[62] Furthermore, serine/arginine rich (SR) proteins are thought to be involved in the alternative splicing of numerous transcripts because their expression levels can influence splice-site selection.[63] These proteins function as driving forces during spliceosome assembly and also play decisive roles in alternative splice-site selection, suggesting that they are crucial players in the regulation of splicing during cell differentiation.[64] Since SR proteins also possess intracellular transport properties, they are also probably involved not only in nuclear pre-mRNA splicing, but also in mRNA transport, cytoplasmic events, and/or mechanisms that involve communication between the nucleus and the cytoplasm.[65] In this context, SR proteins play a crucial role in the selection of FN-spliced domains during development, regeneration, and oncogenesis.[66] Sfrs3 is one of the SR proteins that may selectively exclude or include Fn1 variants [67] during the wound healing process. Moreover, during the wounding process, Fn1 and Sfr3 genes are transcriptionally regulated by one (or more) common transcription factor(s). The alteration of transcription factors/promoter complexes may result in dysfunction of the recruitment and activation of different splicing factors that function to differentially select FN spliced domains for wound repair.

### DNA modifications

PARylation is a covalent protein modification, regulating wound healing process,[68] carried out by poly (ADP-ribose) polymerase (PARP) enzymes. PARPs cut off nicotinamide from NAD+ and attach the remaining ADP-ribose units to suitable protein acceptors. By cleaving many NAD+ molecules, and adding further ADP-ribose units to the protein-proximal first residue, these enzymes build a large, branched poly (ADP-ribose) (PAR) polymer on proteins. PARP enzymes are activated in the presence of DNA damage, such as breaks, and by oxidative stress-induced DNA damage. However, PARP enzymes also regulate the wound healing process through the PARylation mechanism. El-Hamoly et al.[68] reported that a topically applied PARP enzyme can accelerate wound closure in an excision wounding mouse model. Immunofluorescent analysis for PARP enzyme revealed an increased synthesis of the enzyme in the wound bed.[68] *In vitro* studies evidenced a significantly faster migration of keratinocytes when treated with the PARP enzyme and a significant increase of cytokine inflammatory mediators, such as TNFα, IL-1 and IL-6, when compared with non-treated control cells. These results suggest that the PARylation by the PARP enzyme facilitates the wound healing process by stimulating the synthesis of inflammatory mediators and the activity of keratinocytes. A possible hypothesis for this mechanism is related to the protective role of PARPs

enzymes, which are activated by oxidative stress and DNA damage that potentially are present in tissue damage, including wounds.[69]

cDNA microarrays provide expression analysis of thousands of genes simultaneously, of both epidermal and dermal cells, in normal and pathological conditions. Cole et al.[14] determined the gene expression profile of human skin immediately following injury, using cDNA microarrays (Table 1). Samples of normal and injured skin (epidermis and dermis) were collected, from 30 minutes to one hour following incision, from five healthy females undergoing breast reduction surgery. RNA was extracted, reverse transcribed into cDNA and hybridised onto high-density cDNA microarray membranes containing 4000 genes. Results showed that at 30 minutes, the injury resulted in a consistent increase in gene expression of 124 out of 4000 genes (3%). These genes were mainly involved in transcription and signalling. None of the 4000 genes were decreased at 30 minutes. At one hour, only 46 out of the 4000 genes were increased in expression (1.15%) but 264 out of 4000 (6.6%) genes were decreased by more than two-fold, indicating a silencing of many structural genes. Identified genes were suppressors of cytokine signaling (SOCS-1), rho HP1, and BB1 (the gene encoding for the Ig kappa chain variable segment), that are highly expressed after injury and may have an unappreciated role in regulating the initial inflammatory response (Table 1).

### Mitochondrial activity and oxidative stress

Another key mechanism regulating wound healing is oxidative stress. Evidence suggests that compromised wound healing is related to excessive oxidative stress.[70] The leukocyte NADPH oxidase (Nox) is one of the major sources of ROS involved in pathogen killing, VEGF signalling, and the TNF response.[70] In addition, mitochondrial ROS (mtROS) are relevant in different steps of wound healing. mtROS promote actin-based healing of epithelial wounds in different animal models.[71] In vertebrates, mtROS are involved in the antibacterial activity of macrophages.[72] mtROS also regulate the endothelial cells migration related to VEGF signalling.[73] These results suggest the development of specific antioxidants targeting the mitochondria.[70] For example, mitochondria-targeted cationic derivate of coenzyme Q (MitoQ), vitamin E (MitoVitE), and SOD-mimetic TEMPO (MitoTEMPO) prevent cardiac dysfunction induced by ischeamic reperfusion, septic inflammation and endothelial dysfunction.[70] *In vivo* experiments showed that mitochondria-targeted plastoquinones ($SkQ_1$ and $SkQR_1$) prevented nephropathy and brain damage induced by ischaemic injury, and pyelonephritis.[74] $SkQ_1$, *in vitro*, was effective in activating TGFβ and the subsequent myofibroblasts formation.[75] $SkQ_1$ also stimulated *in vitro* wound closure in monolayers of fibroblasts and epithelial cells.[76] Demyanenko et al.[70] demonstrated that the $SkQ_1$ treatment accelerated the inflammatory phase, the formation of the granulation tissue, angiogenesis and re-epithelisation of the wounds, in an excision wound mouse model. Histological analysis revealed an increased amount of myofibroblasts involved in the deposition of ECM proteins and growth factors regulating the granulation tissue formation. $SkQ_1$ also stimulated fibroblasts to synthetise TGFβ, which targets the motility of endothelial cells *in vitro*, and promotes angiogenesis.

### Bacteria

Intestinal microbiota influence both normal and pathological mechanisms in humans, especially in the gastrointestinal tract.[77] For example, commensal bacteria in the colon produce key vitamins and nutrients that regulate metabolism.[78] The challenge for researchers and clinicians consists in determining if intestinal microbiota may be effective in the prevention of intestinal infections developing into a longer-term disease state and/or in therapy. In wound healing, there is evidence that microbes are involved in delayed wound healing, although this association does not always exist.[79] Specific bacteria strains (such as *Staphylococcus*, *Streptococcus* and *Pseudomonas aeruginosa*) produce potent virulence factors and proteases that destroy tissue and impair healing.[80] Chronic wounds have alteration in microflora, such as reduction of bacterial diversity and more opportunistic organisms, in comparison with normal skin.[81] Experimental studies in small and large animals investigated the mechanisms regulating biofilm activity. In a mouse model, researchers created *Staphylococcus aureus* or *Staphylococcus epidermidis* biofilms on open wounds and demonstrated a delay in wound re-epithelialisation that was directly correlated with biofilm formation.[82] *Pseudomonas aeruginosa* biofilms in diabetic mouse wounds prolonged inflammation, tissue necrosis, and delayed healing.[83] Levkovich et al. discovered that female mice fed with the lactic acid bacterium *Lactobacillus reuteri* show more frequent grooming activity compared with controls.[84] Grooming is mainly regulated by the oxytocin hormone, which has a key role in mammalian parturition and lactation. However, recently, it has been hypothesised that oxytocin is also involved in phenomena such as body-energy balance[85] and regulation of the immune system.[86] This suggests the presence of a 'microbiome-gut-brain axis',[87] where probiotic organisms initiate immune-related and neural signals that are transmitted from the gut to the central nervous system, either through blood circulation or directly via the vagus nerve.[88] In this context, it has been discovered that *Lactobacillus reuteri* promotes wound healing through upregulation of the neuropeptide hormone oxytocin, by a vagus nerve-mediated pathway. Specifically, in naive Rag2-deficient animals, lactobacilli activate CD4+Foxp3+CD25+ immune T regulatory cells, promoting wound healing.[89]

The knowledge of a DNA-associated mechanism regulating wound healing may be helpful in developing new strategies in diagnosis and treatment. In addition, increasing knowledge of the epigenetic mechanisms in wound healing may prove useful for developing treatments that are more effective. At present, stem cell-based regenerative medicine holds great promise for wound healing treatments.[90] In fact, epigenetics involves signals that give stem cells their particular abilities to self-renew and differentiate into different damaged cell types.[91]

Nutrition, considered an environmental signal, may be an epigenetic factor regulating the genetic wound healing mechanism. However, experimental and clinical evidence supports nutritional supplementation for promoting the healing of wounds, although the exact molecular mechanism of action of micronutrients and macronutrients is not well understood.[92] To support this hypothesis, there is also the evidence that malnutrition negatively interferes with wound closure, suggesting a putative role of specific nutrients in the regulation of key genes and proteins of the healing pathway.[93]

## Conclusions

DNA provides signatures for the recruitment of histone-modifying enzymes and regulatory transcription factors, as well as information for the control of expression of microRNAs that influence cellular activity. The molecular cell response to trauma and infection is not well explained, but we know that this involves activation of post-translational and epigenetic mechanisms. The biochemical flow chart of biochemical signals, and the remodelling of the transcriptome and, consequently, the influencing of the cell phenotype, is variable by individual, and depends not only on environmental and lifestyle factors, but also on hereditary features.

Future research must concentrate on the genetic, biochemical and physiological differences of the acute and chronic wound, and the interaction with specific nutritional supplements, and local therapies or advanced medications. The application of engineered cells, tissues, and synthetic materials is based on simulating the gene and protein activity of the wound. The integrated knowledge of both the therapeutic approaches for promoting the closure of wounds may be relevant for the management of chronic wounds, which are resistant to common therapeutic protocols, probably because of the deficit of some individual genetic pathway.

It is important for a clinician to integrate genetic, epigenetic, biochemical, regenerative, biotechnological and nutritional evidence to achieve a full knowledge of the complex wound healing mechanism.

We are not completely aware of the molecular algorithm of wound restoration, but we are progressively adding precious information to this puzzle with direct feedback in the clinical setting. **JWC**

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**Reflective questions**

- Summarise biotechnologies used to investigate molecular mechanisms in wound healing.
- Describe in which steps epigenetic interferes with wound healing mechanism.
- What are the molecular mechanisms counteracting wound healing? What procedures should be adopted to reach this goal?


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