Models for the study of skin wound healing. The role of Nrf2 and NF- κ B

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Nrf2 and NF- κ B transcription factors act in wound healing via their anti-inflammatory and anti-oxidant effects or through the immune response. Studying this process is a matter of some importance given the high cost of wound treatment. A major contribution in this regard is being made by models that enable investigation of the involvement of multiple factors in wound healing and testing new curative substances. This literature review was carried out via searches in the PubMed and Web of Science databases up to 2016. It covers skin wound healing, available models for its study (part I), the role of Nrf2 and NF- κ B, substances that influence them and whether they can be used as markers (part II). Was found that *in vitro* assays are used for their availability but a holistic view must be established *in vivo*. *In silico* approaches are facilitating assessment of a vast amount of research data. Nrf2 and NF- κ B play a crucial and reciprocal role in wound healing. Nrf2 controls repair-associated inflammation and protects against excessive accumulation of ROS while NF- κ B activates the innate immune reaction, proliferation and migration of cells, modulates expression of matrix metalloproteinases, secretion and stability of cytokines and growth factors for wound healing.

Key words: skin wound healing, *in vitro* and *in vivo* models, Nrf2, NF- κ B

Received: September 13, 2016; Accepted with revision: December 13, 2016; Available online: January 6, 2017
<https://doi.org/10.5507/bp.2016.063>

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INTRODUCTION

According to the data, between 1 and 2% of people experience a chronic wound during their lifetime¹ and this number will grow due to the rapid rise in the elderly population² because the prevalence of non-healing wounds is highly correlated with age³.

The most serious work now is the testing of new drugs and substances that can improve wound healing⁴ based on knowledge of the general kinetics of normal healing as well as under various pathological conditions (e.g. diabetes mellitus⁵, immunosuppressed hosts⁶ etc.). These facts are also used in forensic medicine for determining the age of lesions⁷. Wound healing components are tested as solutions, gels and ointments or in more complex wound care structures (e.g. hydrogel systems^{8,9}).

WOUND HEALING AND ITS STUDY

Wound healing is a process which ensures the restoration of skin integrity. Intact skin is necessary for almost all of its functions; especially its primary function as a protective barrier against the environment¹⁰, which prevents the entry of microorganisms that can cause non-healing wounds. There are three phases of wound healing: inflammation, proliferation and remodelling (see Fig. 1), although sometimes four are mentioned when coagulation to stop bleeding after injury is considered a separate phase.

The first phase, which starts after injury, is inflammatory. It is characterised by reactions mediated by cyto-

kines, chemokines and growth factors. These substances are produced by cells that migrate to the wound area¹¹ and afterwards cell proliferation, migration and differentiation are activated¹². It can be divided into the early (1-2 days after injury) and the later (days 2-3) inflammatory phase. While in the early phase, the complement is activated and leads to the infiltration of granulocyte neutrophils, blood monocytes changing into tissue macrophages is typical of the later phase¹³. The task of this phase is initiating wound repair.

During the second – the proliferation phase – fibroblasts migrate inward from the edges of the wound and produce proteoglycans, glycosaminoglycans and collagens which create granulation tissue. The migration is activated by basic fibroblast growth factor (bFGF) and transforming growth factor (TGF) produced by macrophages and platelet-derived growth factor (PDGF) from platelets. It is also chemotactic¹⁴. In this phase keratinocytes and endothelial cells also proliferate¹⁵.

The final phase is remodeling and contraction of the wound, which starts after the formation of the granulation tissue. Conversion of the granulation tissue to scar tissue is accomplished by remodeling of the extracellular matrix¹⁶. Wounds never reach the same level of tissue strength¹⁷.

WOUND HEALING MODELS FOR THE SKIN

Wounds can be divided according to their healing time into acute and chronic¹⁸, where the healing process lasts longer than 12 weeks after the initial insult¹⁷. Another classification can be according to the mechanism of origin

Fig. 1. Phases of wound healing: processes, cells and factors involved.

FGF – basic fibroblast growth factor; ECM – extracellular matrix; PDGF – platelet-derived growth factor; TGF – transforming growth factor.

(mechanical – e.g. laceration, puncture, bruise; chemical – acids and alkalis; thermal – burns, scalds, frostbites; actinic – radiation) or the depth of the wound (superficial and deep) (ref.¹⁹). Special models exist for all these types of wounds and for healing under many unusual conditions, e.g. diabetes mellitus^{20,21}, transplantations²², skin grafts, cutaneous ischemia and reperfusion injury, ulcers, decubitus²³, foreign bodies²⁴, and stress situations²⁵.

In this review, wound healing models for the skin are divided according to the design of the experiment into: *in vitro*, *in vivo*, *ex vivo* and *in silico*.

In vitro assays

In vitro models are created from various types of the primary cell cultures or cell lines, of animal or human origin. The most often used are skin keratinocytes and fibroblasts. For more complex models, endothelial cells, macrophages²⁴, melanocytes and Langerhans cells^{26,27} are also utilized. Primary human dermal cells are cultivated from the skin of healthy donors who undergo surgical intervention (plastic surgery, circumcision) or directly donate a skin specimen²⁸. From specific layers of skin tissue explants skin keratinocytes (epidermis and stratum corneum), fibroblasts (dermis) and melanocytes (split-thickness skin) (ref.²⁷) can be isolated using distinctive protocols. Primary endothelial cells are isolated from the umbilicus (human umbilical vein endothelial cells - HUVEC) or the foreskin (human dermal microvascular endothelial cells) (ref.²⁹). Macrophages can be obtained from blood monocytes or rarely from bone marrow³⁰ and Langerhans cells are derived as a subpopulation of dendritic cells from CD34⁺ haematopoietic progenitors due to granulocyte macrophage-colony stimulating factor and tumour necrosis factor- α (ref.²⁷). Among the human cell lines used in these models are e.g. immortalized human skin keratinocytes (HaCaT) (ref.^{31,32}) and endothelial cells (e.g. EA.hy926) (ref.⁴). The animal cell lines include mouse fibroblasts L929 (ref.³²) or Balb/3T3 (ref.³³), keratinocytes (XB-2) as well as their progenitors (MPEK-BL6) (ref.³³) or the murine macrophage RAW264.7 (ref.^{33,34}). The main advantages of the cell lines are that all cells have

the same features and can be cultivated for a long time. On the other hand, immortalized cells are transformed and there are deviations from the normal cells³⁵, whereas the primary cells have various parameters that depend on the donor (e.g. age, gender and ethnicity) and enable a lower number of passages.

In vitro models could also be created from several kinds of cells (co-cultures). The simplest co-cultures are constructed from primary dermal fibroblasts and primary epidermal keratinocytes³⁶ or HaCaT (ref.³⁷). The keratinocytes have to be seeded on a collagen-coated surface or on a cell monolayer e.g. fibroblasts³⁸, HUVEC or both³⁹. These elementary co-cultures are one of the ways to prepare a model that is closer to actual skin. Approaches using stem cells for wound healing improvement seem to also be very promising, because these cells excrete many healing mediators into the culture medium such as TGF- β 1, PDGF, bFGF, keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), interleukin (IL-) 6 and IL-8 (ref.^{32,40}). Stem cells can be used indirectly through conditioned medium^{32,40,41} or directly co-cultured with e.g. dermal fibroblasts⁴⁰ up to more complex structures such as the dermis⁴². In both cases positive effects on wound healing were observed^{32,40,41}.

Another possibility is directly preparing a three-dimensional (3D) skin model. This model can be created from one type of cell or from co-cultures^{37,43}. The 3D structure is achieved with special scaffolds (hydrogel⁴⁴, collagen matrices and lyophilized membranes, inert filters or de-epidermized dermis⁴²). Commercially available kits can be used to prepare the 3D structure that include a special surface for seeding the cells (EpiDermTM or EpiSkinTM for keratinocytes that represent the epidermis or EpiDermFTTM that includes fibroblasts as the dermis²⁸). Some models of reconstructed human skin can also be populated with Langerhans cells and/or melanocytes. These more complex models are used for studying cell – cell or cell – matrix as well as dermal – epidermal interactions⁴² and observing cell migration in structures similar to the extracellular matrix⁴⁵. Other fields of study are the response of cells to pharmacological agents, their

growth and other biological factors and their effect on wound healing⁴². A great benefit of the artificial skin constructs is the possibility of testing ultraviolet (UV) radiation⁴⁶ or irritating noxae and obtain initial data without using an animal⁴⁷.

The advantages of *in vitro* models are price (they are relatively cheap), simplicity and the ethical considerations that are not as complex as with *in vivo* models. Inherent heterogeneity can be excluded when the same cells are used, as can changes in the environment²⁴ and the influence of the entire organism²⁶. Their disadvantage is the problem of extrapolating results to the whole organism²⁴.

In vivo assays

In vivo models are more expensive, demanding and involve many ethical considerations compared to *in vitro* ones, but the results with a holistic approach are often irreplaceable²⁴. It makes it possible to evaluate the interplay of the host immune response with external intervention that is crucial in contaminated wounds⁴⁸. Sometimes the factors have different effects *in vivo* and *in vitro* – e.g. TGF- β promotes angiogenesis *in vivo* while *in vitro* it inhibits proliferation and the growth of endothelial cell monolayers. There are more explanations, but one of them suggests a connection to macrophages that produce other angiogenesis-stimulating factors⁴⁹.

In vivo assays can be divided into animals and humans. Animals are used more often because only small and clean wounds²⁴ can be created in humans, usually using the sun-protected area of the forearm on a non-dominant limb⁵⁰. With this arrangement, two wounds can be made, in an ideal case one for each arm, and one of them can serve as a control²⁴. If we ignore the fact, that there are differences between human and animal skin, testing on people has another invaluable benefit: the tested person can evaluate pain during the wound process. Depressive

symptoms are too difficult to measure in animals⁵⁰ and humans are also better for assessment of psychological stress, although it is possible test stress in e.g. mice²⁵.

Because most experiments aim to extrapolate to humans, mammals are usually used, although e.g. chicken chorioallantoic membrane can be used for angiogenesis assays⁵¹ and the effect of stress on wound healing has been investigated in lizards⁵². Rats, mice, pigs and rabbits are the most frequently used animals. Hamsters²⁴, dogs, cats, sheep, horses and ponies⁵³ are also utilized. Porcine skin is the closest to human, due to its composition²⁴ as well as its tight skin type and mechanism of wound closure (only re-epithelization) (ref.⁵³). For example, rodents have a special muscle *panniculus carnosus* that facilitates healing due to wound closure contraction, and that is why mice, rats, hamsters and rabbits heal more rapidly than humans^{24,53}. To avoid this phenomenon, scientists make a wound e.g. on rabbit ears where the contraction does not occur²⁴. There are many differences between the thickness of particular layers of human and animal skin and there is also a large variability in the number of hairs per cm² (ref.^{54,55}) (see Table 1). When selecting between mouse and rat, rat skin strata and whole skin thickness are closer to human than those of mice⁵⁶. Knowledge of interspecies and intraspecies differences helps with the interpretation and extrapolation of results between species⁵³.

Animal species as well as individuals within a species differ in size, and that is why animals are characterized by weight. The number and the area of wounds that can be made depend on the animal's size and the part of the body. The smallest wounds, on the order of several-mm punches, can be made in mouse embryos⁵⁷, round wounds up to 2 cm in diameter are possible in rats⁵⁸, and in pigs wounds with areas of tens of square centimetres can be studied⁵⁹. The biggest free surface is the dorsum, which is also difficult for an animal to reach, and that is why

Table 1. Comparison of skin composition between human, domestic pig, hairy mouse, hairy rat and rabbit^{24,53-56,141-154}.

Thickness of the skin layers (in μm)					
Part of skin	Human (Ref.)	Domestic pig (Ref.)	Hairy mouse (Ref.)	Hairy rat (Ref.)	Rabbit (Ref.)
Stratum corneum	10-20 (56,149)	10-15 (54,147)	2.9-9 (54,146,147)	3-30 (54,56,147,148)	4-11 (55,147)
Epidermis	30-130 (56,149)	50-100 (54,147)	9-29 (54,56,143,146)	11-32 (56,144,147,148)	11-20 (55,147)
Dermis	800-1500 (149)	514-3000 (141,145)	260-280 (143,146)	350-810 (148)	433-1600 (152,154)
Hypodermis	1200 (149)	2810-5000 (141,150)	80-105 (143,146)	400 (144)	950 **
Whole skin*	2-3 (54,56)	2-8 (142,151)	0.4-0.7 (54,56)	0.85-1.45 (148,153)	1.4-2.6 (55)
Other skin characteristic					
Paniculus carnosus ^{24,53}	no	no	yes	yes	yes
Hairs/cm ²	11 (54)	11 (54)	658 (54)	289 (54)	4700-7500 (55)

* In mm

** The value for rabbit hypodermis was determined from values for whole skin and other layers (this information was not found in the literature).

this area is often used²⁴. Pigs are also very useful because their size enables multiple wounds to be studied together on the same animal (control and tested influence or dose and time-dependent experiments) (ref.⁶⁰).

Special models exist for each part of the healing process as well as for various conditions²⁴ such as impaired wound healing on diabetic and fat animals^{21,61}. In particular mice are highly relevant for skin biology studies because many human disease models, knockout strains and transgenic tools can be used with them²³. Hairless or nude animals provide a significant advantage for some research in that it is easier to make and observe a wound, while a hairy animal has to be shaved first. However, the architecture of hairless mouse skin is quite different from humans²⁴. Besides the selection of the correct animal model, good laboratory practice must not be overlooked – animals have to be acclimatized after their acquisition for a few days before the experiment starts, the wound must be inflicted under general anaesthesia⁶⁰ and the animal must be kept clean and watched carefully. Finally, it must be noted that current legislation in the European Union, Israel, India and the State of São Paulo in Brazil only allows drugs for wound healing to be tested on animals, and there is an ongoing search for new approaches that do not have to rely on animal studies⁶².

However it is not only research projects that can provide new information about wound healing issues. Clinical vet practice may also have a significant contribution to make in this field. Especially with dogs and cats, vets treat many kinds of wounds and injuries that are also problems for humans, such as thermal injuries, surgical site infections, pressure ulcers and ischemic wounds but also ballistic and degloving injuries, endocrinopathies and immunosuppression, impaired healing and oncological treatment. And of course animals are great examples for studying bite wounds⁵³.

Ex vivo assays

At the interface between *in vitro* and *in vivo* models are *ex vivo* approaches. Full thickness skin explants (biopsies and then skin tissue culture) can be obtained from animals and humans, both living and dead. Even if we can take larger sections of skin from dead organisms, harvesting from a living body enables the samples taken to be immediately processed and so avoid post mortem changes. The use of laboratory animals is a great advantage, since they can be sacrificed when the researcher needs to start an *ex vivo* experiment with skin. From living humans either small pieces can be obtained thanks to volunteer donors⁶³ or larger sections of skin when a person undergoes plastic reduction surgery (mammo- or abdominoplasty). These *ex vivo* models offer a simple and cost-effective viable design for studying wound healing while preserving epitopes for translation⁶⁴.

The collected skin is usually disinfected with ethanol, freed from subcutaneous fat, rinsed with physiological solution and placed on a suitable surface^{64,65} such as dishes coated with collagen I (ref.⁶⁴) or onto inserts⁶⁶. The wounds are then created and exposed to the tested

agents (cytokines, matrix metalloproteinase inhibitors, silver, natural compounds etc.) (ref.^{64,66}). The viability of cultured explants can be evaluated e.g. by their lactate dehydrogenase activity⁶⁴. When the explants are obtained from volunteer donors preventive effects on wound healing can also be examined – a substance is topically applied to the skin before harvesting⁶³. There are many specialised methods that evaluate the influence of stretching on healing⁶⁷, or angiogenesis and blood vessel permeability during the restorative process (*ex vivo* perfusion) (ref.⁶⁸). *Ex vivo* models can also be very useful for studying the changes in a transdermal delivery system after an injury or for forensic practice, where it is essential to determine the age of a wound and distinguish between a wound created ante- or post-mortem¹⁰.

In silico assays

As the amount of knowledge about wound healing increased, theoretical models were developed^{69,70}. These are primarily used in the research field. It has been found that all chronic wounds have notably comparable healing paths and also similar inflammatory profiles. In the pharmaceutical industry, these findings may result in the determination of new targets for curative drugs. For clinical practice, *in silico* approaches will perhaps provide options for personalized treatment if the model is improved with individual genetic data⁷¹.

THE POSSIBILITIES OF WOUND FORMATION AND EVALUATION METHODS

One of the most important things to ensure is that all tested wounds should be uniform in location, diameter and depth, and also the conditions during wound creation should always be the same. It is easier to create an acute wound than a chronic one. The defect is usually made mechanically (cut, scratch) and/or thermally (heat or cold, radiation). Also chemicals such as acids (sulphuric, triacetic...), alkalis (sodium hydroxide) and nitrogen mustard could be used⁷². While mechanical damage enables observation of re-epithelialization, chemical wounds can be used to study stromal activation⁷³. Sometimes it is useful to cover lesions to prevent them from drying out⁴¹.

Chronic wounds models usually combine acute wound creation and then the injury is exposed to some sort of long-term effect. For *in vitro* applications, a collagen gel matrix with serum protein which mimics the wound bed of a chronic lesion can be used and e.g. bacterial infection studied⁷⁴. *In vivo* true chronic wound models in animals are very difficult to establish because chronic defects do not occur in them and the majority of *in vivo* information about non-healing wounds are obtained from clinical practice with humans⁷⁵. The use of these models lies in bacteriology testing when the particular bacterial strains or their lipopolysaccharides are applied to rodent skin for several weeks and scientists observed the immune system response⁷⁶ or bacterial film formation on a chronic thermal injury⁷⁷. Other research is engaged in ischemic

Table 2. Markers for particular phases and processes in wound healing^{12-14,17,155}.

Phase	Process	Involved molecules that can be used as markers
Inflammatory	Inflammation	IL-1, IL-6, IL-8, IL-10, NF- κ B, Nrf2, MMP-2, HGF, PDGF, TGF- β , TNF- α
	Cell migration	EGF, HGF, KGFs, NF- κ B, NGF
	Cell differentiation	IL-10, EGF, FGFs, TGF- β
	Immune response	IL-10, IL-10, IL-27, PDGF, TGF- β , G-CSF, TNF- α
Proliferative	Proliferation	IL-1, IL-10, EGF, FGFs, IGF-1, KGFs, NF- κ B, NGF, PDGF, TGF- α , TGF- β , G-CSF, GM-CSF, TNF- α
	Reepithelization	IL-1, IL-6, EGF, FGFs, HGF, NF- κ B, PDGF, TGF- α , TGF- β 1
	Angiogenesis	IL-1, IL-6, MMP-2, FGFs, HGF, TGF- β , VEGF
	ECM tissue formation	IL-4, IL-27, IGF-1, PDGF, TGF- β
	Granulation tissue formation	EGF, FGFs, HGF, TGF- α , TGF- β
Remodeling	ECM remodeling	IL-1, IL-6, MMPs, TIMPs, PDGF, TGF- β
	Wound contraction	IL-8, IL-10, FGFs, NGF, TGF- β
	Scarring (fibrosis)	MMP-2, MMP-9, TIMP-1

ECM – extracellular matrix; EGF – epidermal growth factor; FGFs – fibroblast growth factors; G-CSF – granulocyte-colony stimulating factor; GM-CSF – granulocyte macrophage-colony stimulating factor; HGF – hepatocyte growth factor; IGF-1 – insulin-like growth factor; IL – interleukin; KGFs – keratinocyte growth factors; MMP – matrix metalloproteinase; NF- κ B – nuclear factor- κ B; NGF – nerve growth factor; Nrf2 – nuclear factor-erythroid 2 (NF-E2)-related factor 2; PDGF – platelet-derived growth factor; TGF – transforming growth factor; TIMP – tissue inhibitor of metalloproteinases; TNF- α – tumour necrosis factor- α ; VEGF – vascular endothelial growth factor.

wounds e.g. on rabbit ears (the nutrient arteries are divided and an incision wound is created) (ref.⁷⁸). These models can also help with testing therapeutic strategies⁷⁴.

The wound and study of the healing process can be observed morphologically. The area, depth and scar formation of the wound are monitored by cell migration, neovascularization and possible deposition of the tested substance (e.g. silver) (ref.^{63,64,66}). The re-epithelization and scar formation are measured by the incorporation of 5-bromo-2-deoxyuridine⁶⁵. During inflammation, blood vessel permeability increases, so it is also useful to observe this *in vivo* (in mice by intravenous injection with fluorescein isothiocyanate dextran⁶⁸). When a new compound is tested for sanative effects, we must not neglect viability tests such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or neutral red assay or the determination of ROS generation as an oxidative stress marker.

However, today it is almost inevitable that each process is studied at the level of mRNA as well as protein expression. Typical markers are known for the inflammation, proliferation and remodeling phases in general or for more concrete processes (coagulation, immune response and cell activation and differentiation, proliferation, re-epithelization, angiogenesis, extracellular matrix remodeling, epidermal-mesenchymal interaction⁶⁵ etc.). Table 2 provides a brief overview of the habitually studied molecules regulating various wound healing processes and which can be used as markers for the skin restoration process.

There are many ways in which wound healing can be evaluated and thus determine the effect of the compound/treatment method or its influence in general. Briefly, molecular methods and the determination of markers are necessary when a new compound is being studied, while in experiments closer to clinical practice (e.g. the testing of healing systems such as hydrogels, biodegradable materials, scaffolds) the histology becomes more important

(a lesion's area or thickness and the overall morphology of healing).

THE ROLE OF NRF2 AND NF- κ B, AND INTERCONNECTIONS BETWEEN THEM DURING WOUND HEALING

Scientists are currently searching for interconnections between various processes to obtain a deeper understanding of skin restoration. Transcription factors Nrf2 and NF- κ B are now frequently studied for their key role in oxidative stress and inflammatory reaction. The complicated network of their regulatory effects is also involved in wound healing. A summary of their impact on this field and evaluation of their use as markers is given below.

Nrf2 in the wound healing

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a cytosolic transcription factor. Under non-stressful conditions, it is sequestered by Kelch-like ECH-associated protein (Keap1) which marks Nrf2 for proteasomal degradation^{79,80}. In cells under harmful stress, Nrf2 is separated from its inhibitor protein and translocates into the nucleus^{79,81-83}. In the nucleus Nrf2 binds to the antioxidant response elements (ARE), which requires heterodimerization with the small Maf proteins⁸⁰. ARE are located in the promoter region of genes that code many antioxidant and phase II detoxifying enzymes. These enzymes are important in cellular defence by enhancing the removal of cytotoxic electrophiles and ROS (ref.⁸⁴).

The up-regulated expression of Nrf2 (see Fig. 2) was observed⁸⁵ upon skin injury. The main function of Nrf2 during wound healing is protection against the excessive accumulation of endogenous ROS, which are produced

Fig. 2. Nrf2 pathway in wound healing.

ARE – antioxidant response elements; FGF – fibroblast growth factor; GCL – glutamate cysteine ligase; GPX – glutathione peroxidase; GST – glutathione S-transferase; HO-1 – heme oxygenase-1; Keap1 – Kelch-like ECH-associated protein; KGF – keratinocyte growth factor; MAPK – mitogen-activated protein kinases; Nrf2 – nuclear factor-erythroid 2 (NF-E2)-related factor 2; NQO1 – NAD(P)H quinone oxidoreductase; P – phosphorylation; PKC – protein kinase C; PI3K – phosphatidylinositol 3-kinase; PrXI – peroxiredoxin I; ROS – reactive oxygen species; TGF – transforming growth factor; U – ubiquitination.

in large amounts in wounded and inflamed tissues. It was found that there is a pronounced expression of Nrf2 in macrophages and keratinocytes localized in hyperproliferative wound epithelium. Prolonged inflammation was found in knockout animals⁸², although it was not observed to affect skin morphogenesis. This means that Nrf2 plays an important role during the resolution stage of inflammation⁸⁶, but not in the re-epithelization of skin wounds^{81, 87}. Thus Nrf2 has been identified as controlling repair-associated inflammation under physiological conditions. This factor is also irreplaceable in diabetic wound healing because the tissues of a patient with diabetes mellitus already exhibit higher oxidative stress. This stress further increases after injury, and it has been established that pharmacologically elevated levels of Nrf2 are beneficial for impaired wound healing in diabetes mellitus⁸⁸.

Nrf2 inducers

The Nrf2 pathway is activated under stress conditions, especially by ROS (ref.⁸⁹) and ARE inducers. These inducers can be endogenous (NO, H₂O₂, 4-maleyl-acetoacetate, 4-hydroxynonenal, nitro-oleic acid, 15-deoxy-delta-12,14-prostaglandin J2) or exogenous, including phytochemicals (quercetin, oleanolic acid, genistein⁹⁰, resveratrol, sulphoraphan⁸⁹, curcumin⁹¹, zerumbone⁹², 4-ketopinoresinol⁹³), therapeutics (e.g. nimesulide, acetaminophen) and environmental agents (paraquat, metals

– Cd²⁺, Hg²⁺, Au(I), silica, nanotubes) (ref.⁹⁰). The gene encoding Nrf2 is regulated by KGF (cytoprotective factor for epithelial cells) (ref.⁸²) whose level increases after wounding⁹⁴. The expression of Nrf2 in keratinocytes is also induced by dermal fibroblasts through the FGF, especially FGF-7 and FGF-10 (ref.⁹⁵). The stabilization of Nrf2 (decrease the rate of degradation) is promoted by heme, which can be released from heme-proteins under stress conditions such as tissue damage or cell injury⁹⁶, and due to its reactive iron molecule and lipophilic character could have harmful pro-oxidant effects⁹⁷.

Nrf2 inhibitors

Under physiological conditions, the Nrf2 pathway is regulated by the Keap1 protein that sequesters it in the cytoplasm as was mentioned above and also by post-induction repression. In this case, Keap1 enters the nucleus, removes Nrf2 from ARE and exports it back to the cytoplasm. There the ubiquitin machinery Cullin-3 marks Nrf2 for proteasomal degradation^{98,99}, which is related to the diminution of oxidative stress – re-entry to a normal reducing environment with the restoration of normal Nrf2 degradation⁹⁶. Further, E-cadherin inhibits this pathway because it blocks Nrf2 translocation into the nucleus and also TGF- β and some nuclear hormone receptors negatively interfere with it¹⁰⁰. When scientists want to evaluate whether the compound acts through the Nrf2

pathway, they can suppress it via several kinase inhibitors (without phosphorylation, Nrf2 is retained in the cytosol with Keap1) (ref.⁹³), Maf-lacking⁸¹, selective bZip domain complex inhibitors (impede interaction between Nrf2 – ARE interaction) (ref.⁹⁰) and RNA interference (Nrf2 translation blocking) (ref.⁹³). However, there are not many Nrf2 inhibitors yet and because some cancer cells exploit the upregulation of the Nrf2-ARE pathway for easier survival, screening for substances with a suppressive effect is now in the interest of scientists⁹⁰. Of natural compounds, brusatol⁹⁰, ochratoxin¹⁰¹ and trigonellin¹⁰² have been identified. During prolonged inflammation which is a consequence of impaired wound healing, associations with many pathological conditions such as fibrosis and abnormal angiogenesis up to neoplasia are observed¹⁰³. And because in stressful environments, the most resistant cells are selected, the overexpression of Nrf2 can also be dangerous, even though it is generally considered to be a protective transcription factor.

NF- κ B in wound healing

NF- κ B (nuclear factor- κ B) monitors the expression of genes involved in inflammatory and oxidative stress response, differentiation, proliferation, apoptosis and cell adhesion¹⁰⁴. In the cytoplasm, NF- κ B proteins are kept in association with inhibitory I κ B proteins that are degraded in response to stress stimuli. This leads to the release and activation of NF- κ B, which can translocate to the nucleus¹⁰⁴⁻¹⁰⁵.

During wound healing, the classical NF- κ B pathway is activated (see Fig. 3) as an innate immune reaction and many cytokines, chemokines, adhesion molecules, enzymes which produce secondary inflammatory mediators, major histocompatibility complex class II antigens and inhibitors of apoptosis are created¹⁰⁵. All these factors (IL-1 β , IL-6, IL-8, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, inducible NO-synthase and cyclooxygenase-2) are essential in the early protective response against pathogens¹⁰⁶. This is why NF- κ B activation occurs in almost all cells¹⁰⁷ during infection or injury¹⁰⁸, especially in macrophages and epithelial cells¹⁰⁹. This factor then becomes necessary for the migration of phagocytic and inflammatory cells to the tissues¹⁰⁹. On the other hand, errors in NF- κ B regulation, especially up-regulation, can cause chronic diseases (rheumatoid arthritis¹¹⁰, psoriasis¹¹¹, diabetes mellitus, vascular complications¹¹², inflammatory bowel disease¹¹³, vascular inflammation and cardiovascular hypertrophy¹¹⁴). Because NF- κ B also controls genes involved in cell proliferation (granulocyte colony-stimulating factor and macrophage colony-stimulating factor¹⁰⁷), transformation and survival (Bcl-X_L, Bcl-2) (ref.¹¹⁵) its anti-apoptotic and proliferative effects can facilitate tumour formation and the creation of metastases¹¹⁶. A key aspect is the stimulatory environment, which partly determines whether the effect of NF- κ B is protective or deleterious for the host¹⁰⁶. During wound healing, its proliferative effect is involved in re-epithelization because it regulates keratinocyte proliferation

Fig. 3. Classical NF- κ B pathway in wound healing.

Bcl – protein family regulates apoptosis; COX-2 – cyclooxygenase-2; G-CSF – granulocyte-colony stimulating factor; I κ B – inhibitory protein of NF- κ B; IL – interleukin; iNOS – inducible nitric oxide synthase; M-CSF – macrophage-colony stimulating factor; NF- κ B – nuclear factor- κ B; P – phosphorylation; ROS – reactive oxygen species; TNF- α – tumour necrosis factor- α ; U – ubiquitination; UV – ultraviolet.

and migration^{12,117}. This transcription factor also controls the expression of matrix metalloproteinases¹¹⁷, which facilitate keratinocyte movement, modulate the secretion and stability of cytokines and growth factors for epidermal wound healing⁶⁵.

NF- κ B inducers

The classical NF- κ B pathway is activated by pro-inflammatory cytokines (IL-1 β , tumour necrosis factor- α (ref.¹⁰⁷), IL-6 (ref.¹¹⁸)), T- and B-cells antigen receptors^{107,109} and angiotensin II (ref.¹¹⁹). Other inducers include viruses, bacterial lipopolysaccharides (activation via toll-like receptors which are linked to the NF- κ B system by different adapter proteins (ref.¹²⁰)), some protozoan parasites (e.g. *Trypanosoma cruzi*), ROS (ref.¹¹²), phorbol ester¹²¹, laser¹²², UV (ref.¹⁰⁷) and ionizing radiation¹²³. There is also an amplification loop – NF- κ B that initiates the expression of IL-6 (ref.¹¹⁸), IL-1 β and tumour necrosis factor- α in the nucleus, and this leads to the other type of activation of NF- κ B in the cytoplasm¹⁰⁹. The indirect NF- κ B pathway results in the release of IL-1 β and activation of the classical NF- κ B pathway in neighbouring cells¹²⁴.

NF- κ B inhibitors

Overexpression of NF- κ B, as well as inadequate action can lead to impaired wound healing. In particular, prolonged inflammation, anti-apoptotic and proliferative effects are all dangerous^{106,116}. That is why this pathway must

be kept within normal limits. NF- κ B can be down-regulated by inhibition of the protein kinases that are necessary for the phosphorylation and polyubiquitination that lead to degradation of its inhibitory subunit¹²⁵. Another way in which NF- κ B activation can be repressed is using phosphatases (phosphatase 2A) (ref.¹²⁶), proteasome inhibitors (dipeptide boronate – bortezomib) (ref.¹²⁷), I κ B ubiquitination blockers (N-tosyl-L-phenylalanine chloromethyl ketone) (ref.¹²⁸), small peptides that cross the cell membrane and block the translocation of NF- κ B into the nucleus (SN50) (ref.¹²⁹), inhibitors of p65 acetylation (histone deacetylases) (ref.¹³⁰) or sesquiterpene lactones that bind to DNA and occupy κ B-specific sites¹³¹. Then antioxidants with other mechanisms of effect (e.g. inhibition of ROS production), special bacterial, fungal¹⁰⁴ and viral proteins¹³² can be used as NF- κ B suppressors. Of the commonly used drugs, anti-inflammatory agents (both steroid and non-steroid) (ref.^{133,134}), selective estrogen receptor modulators such as raloxifene¹³⁵ and immunosuppressive agents¹³⁶ have an inhibitory effect on the NF- κ B pathway. A detailed summary of NF- κ B pathway inhibitors has been written by Gupta et al. (ref.¹⁰⁴).

Relationship between Nrf2 and NF- κ B

To put it very simply, the Nrf2 and NF- κ B pathways work against each other. Nrf2 protects cells against oxidative stress and inflammation, while NF- κ B triggers an innate immune reaction and thus inflammation as well

Fig. 4. The relationship between Nrf2 and NF- κ B.

CBP – CREB binding protein; COX-2 – cyclooxygenase-2; Gai2 – inhibitory G protein; HO-1 – heme oxygenase-1; I κ B – inhibitory protein of NF- κ B; IL-8 – interleukin 8; Keap1 – Kelch-like ECH-associated protein; NF- κ B – nuclear factor- κ B; Nrf2 – nuclear factor-erythroid 2 (NF-E2)-related factor 2; P – phosphorylation; PI3K – phosphatidylinositol 3-kinase; ROS – reactive oxygen species.

as oxidative stress^{100,137}. Nevertheless, there are many interconnections between their pathways. Some common genes such as IL-8, heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit and inhibitory G protein (Gai2) are targets for both transcription factors¹³⁸. The relationship between Nrf2 and NF- κ B is shown in Fig. 4.

Both pathways are activated by oxidative stress (Nrf2 by low and NF- κ B by intermediate amount of ROS) (ref.⁹⁸) and other stimuli that lead to the release of nuclear factors from their repressors. The protein kinase complex that enables the phosphorylation and degradation of I κ B protein (NF- κ B repressor) and therefore NF- κ B activation can be inhibited by the Nrf2 repressor Keap1 (ref.⁹⁸). On the other hand, the NF- κ B subunit p65/RelA co-imports Keap1 into the nucleus, where it terminates the effect of Nrf2 on gene transcription by exporting this factor back to the cytoplasm¹⁰⁰. When both transcription factors reach the nucleus they need CREB binding protein (CBP) co-activators that are necessary for gene transcription. This is why the simultaneous activation of Nrf2 and NF- κ B results in competition for these co-activators^{98,137}. Some of their gene expression targets can also work against each other. NF- κ B activates cyclooxygenase-2 (COX-2), which suppresses phosphatidylinositol 3-kinase and so inhibits the phosphorylation of Nrf2 and its release from Keap1 (ref.^{100,139}). Going the other way, HO-1 activated by Nrf2 can block NF- κ B translocation into the nucleus by inhibiting I κ B protein degradation^{98,138}. However, this information is somewhat inconsistent, because it was found that NF- κ B also has a protective effect against ROS and this can be realized by increasing the expression of antioxidant genes such as HO-1 (ref.¹³⁷). The return of ROS to homeostasis due to the antioxidant genes activated by Nrf2 not only causes a feedback inhibition that leads to a reduction in the activation of this transcription factor itself¹⁴⁰, but also indirectly inhibits the NF- κ B pathway. Consequently Nrf2 prevents the cellular damage of inflammation-exposed cells¹⁰⁰.

Nevertheless, these two pathways may also be activated reciprocally: oxidative stress triggered by NF- κ B backward-activates NF- κ B and also facilitates the translocation of Nrf2 into the nucleus to protect cells against inflammation and oxidative damage^{98,100,138}. Further, Nrf2 can help the release of NF- κ B from I κ B by increasing proteasome activity and thus potentiates the protein degradation of this inhibitory protein¹⁰⁰.

Nrf2 and NF- κ B as possible markers for wound healing

Nrf2 and NF- κ B are important during wound healing. However, it is difficult to specify how these two transcription factors can be used for evaluating wound healing. Under physiological conditions (non-stress, no injury) the levels of these two markers are minimal, so there are no defined "physiological levels" for them. It can be generally said that Nrf2 is a protective factor, but its overexpression can block the necessary immune response and insufficient activation caused prolonged inflammation⁸², cell damage and even neoplastic transformation⁷⁹ due to inadequate antioxidant response. Similarly NF- κ B is nec-

essary for the correct response to infection or injury¹⁰⁸. A lack of it impedes the accurate course of the innate immune reaction, whereas excess production may lead to inflammatory, other chronic diseases^{110-112,114} and tumour formation¹¹⁶.

Every wound is different and needs different defence and repair reactions. That is why it is practically impossible to use these two transcription factors for evaluation in clinical practice, although e.g. zero levels of these transcription factors at large non-healing wounds can be a certain indicator of an inadequate defence against oxidative stress (Nrf2) or innate immune reaction (NF- κ B). The main utilization of Nrf2 and NF- κ B as markers is applied in the testing of new healing compounds. For wounds with prolonged inflammation, compounds that decrease the NF- κ B levels or activate the Nrf2 pathway can be useful. On the other hand in immuno-suppressed patients, NF- κ B induction can help to start the correct immune reaction. Nevertheless, it is necessary to stress that the complicated network of relationships between many active molecules in the whole body can cause a different cell response than in *in vivo* tests.

CONCLUSION

This review briefly summarizes skin wound healing, different kinds of models for its study and the role of the transcription factors (Nrf2 and NF- κ B) in this process and if they can be used as markers.

For researchers dealing with wound healing, it can be really difficult to select the best model for experiments. First of all, they have to know if they would like to study it in general or a particular phase/process. For the initial stages of a study, *in vitro* models are best due to their availability and cheapness. Nevertheless, these arrangements do not allow a holistic view, and thus *in vivo* assays also have to be used, usually on animals. The substances/ influences that appropriately affect skin restoration process can be later used in humans. For a closer approximation to a specific type of wound there are a number of methods for designing and conducting experiments, including various techniques and models that imitate acute and also chronic wounds. For special conditions immunosuppressed, diabetic or various gene knockout animals can be used. *In silico* approaches can help with the assessment and linking of vast amounts of research data.

For evaluating all these experiments, morphologic changes as well as molecules involved in wound healing can be studied and this requires proper selection of parameters and markers. Nrf2 and NF- κ B act via the anti-inflammatory and antioxidant effect or innate immune response in wound healing. The main effects of the above transcription factors are supplemented by many others, and thus create a complicated net with several feedback or amplification loops and interconnections. This is why increase/ decrease in given markers is valueless because it depends on the particular wound environment and state of health of the organism. The crucial activity of these

two transcription factors in wound healing is however unquestionable.

ABBREVIATIONS

ARE, Antioxidant response elements; CBP, CREB binding protein; COX-2, Cyclooxygenase-2; (b)FG, (basic) fibroblast growth factor; HaCaT, Immortalized human skin keratinocytes; HO-1, Heme oxygenase-1; HUVEC, Human umbilical vein endothelial cells; IκB, Inhibitory protein of NF-κB; IL, Interleukin; Keap1, Kelch-like ECH-associated protein; NF-κB, Nuclear factor-κB; Nrf2, Nuclear factor-erythroid 2 (NF-E2)-related factor 2; ROS, Reactive oxygen species; TGF, Transforming growth factor; UV, Ultraviolet.

Search strategy and selection criteria

Our research strategy was aimed at evaluating studies on the role of the transcription factors (Nrf2 and NF-κB) in skin restoration to determine if they can be used as markers. Scientific articles from 1974 to 2016 were searched using the PubMed and Web of Science databases. All searches were up to date as of August 2016. The search terms used included “skin wound healing”, “models for wound healing”, “evaluation of wound healing”, “Nrf2 in wound healing”, “NF-κB in wound healing” and “interconnection between Nrf2 and NF-κB”. Only English language papers were reviewed.

Acknowledgement: This work was supported by the grants from Palacky University (IGA_LF_2015_007 and IGA_LF_2016_012) and by the National Programme for Sustainability I (L01304).

Author contributions: NA: literature search, manuscript writing, figures, tables; AG: concept, critical revision; JU: critical revision.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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