The Neuronal Nitric Oxide Synthase Is Upregulated in Mouse Skin Repair and in Response to Epidermal Growth Factor in Human HaCaT Keratinocytes

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Expression of nNOS mRNA was found in normal human and mouse skin tissue. Upon wounding, we observed a rapid downregulation of nNOS mRNA and protein in wounds of mice; however, when repair continued, nNOS mRNA was strongly upregulated and nNOS protein expression peaked at late stages of healing. Immunohistochemistry revealed wound keratinocytes as the cellular source of nNOS. In line with the *in vivo* situation, we found a basal expression of nNOS in the human keratinocyte cell line HaCaT. A marked stimulation of nNOS expression in the cells was achieved with epidermal growth factor receptor (EGFR) ligands such as epidermal growth factor (EGF), heparin-binding EGF, transforming growth factor-α and two alternate splicing forms of the neuregulin gene. EGF-induced induction of nNOS was completely inhibited by the specific EGFR antagonist PD153035 and by the EGFR and Janus kinase 2/3 inhibitor AG490. Activation of EGFR might contribute to the observed upregulation of nNOS also in skin repair, as we found a spatial and temporal correlation of phosphorylated EGFR (Y1173) with nNOS expression at the wound site. Thus, in addition to the inducible- and endothelial-type NOS isoforms, also nNOS expression is regulated in the process of cutaneous wound repair.

Key words: growth factor/nitric oxide/wound healing
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Nitric oxide (NO) is now well established as an important messenger molecule that is involved in physiological and pathophysiological processes in numerous organ systems. A variety of different cell types are able to release NO, which can be synthesized by three distinct nitric oxide synthase (NOS) isoforms: the neuronal (nNOS), the inducible (iNOS), and the endothelial (eNOS) isoform (Moncada *et al*, 1991; Förstermann *et al*, 1994; Alderton *et al*, 2001).

NO has been shown to play important roles in normal skin biology and also diseased skin (Bruch-Gerharz *et al*, 1998a, b; Weller, 1999; Frank *et al*, 2002). Constitutively expressed, eNOS and iNOS have been initially reported in keratinocytes from normal skin (Shimizu *et al*, 1997). Studies on skin tissue have also demonstrated a key function of NO in the process of cutaneous wound healing (Frank *et al*, 2002). A crucial role for iNOS enzymatic activity during the repair process was suggested, as iNOS-deficient mice suffered from a delay in wound repair (Yamasaki *et al*, 1998), and as inhibition of iNOS during healing resulted in a markedly reduced re-epithelialization (Stallmeyer *et al*, 1999). In line, an early induction of iNOS upon wounding has been well documented (Frank *et al*, 1998a, b).

It is now established that expression of human nNOS is not restricted to neuronal cells. Interestingly, recent studies have demonstrated the expression of the neuronal isoform also in skin-derived cell types such as keratinocytes and melanocytes (Baudouin and Tachon, 1996; Romero-Graillet *et al*, 1996; Ormerod *et al*, 1998). Moreover, nNOS-derived NO has been shown to be involved in the modulation of skin proliferation and melanogenesis (Romero-Graillet *et al*, 1997).

Initially, the nNOS protein was considered to be constitutively expressed; however, more recent evidence demonstrated that expression of the nNOS gene can be regulated by a variety of physiological and pathological stimuli (Dawson *et al*, 1998; Förstermann *et al*, 1998). Accordingly, the human nNOS gene exhibits a complex expressional regulation, with multiple nNOS mRNA species produced in a tissue-specific and developmentally regulated manner (Wang *et al*, 1999; Saur *et al*, 2002; Boissel *et al*, 2003). In this study, we investigated the presence and potential regulation of nNOS mRNA and protein expression in skin repair. Interestingly, we found the nNOS isoform expressed in keratinocytes at the wound site. Moreover, *in vitro* studies on keratinocytes showed that upregulation of nNOS expression was restricted to stimulation of the epidermal growth factor receptor (EGFR). Thus, our data suggest an expression of nNOS in the epithelial compartment during skin repair that is most likely under control of EGFR-directed signaling pathways.

Abbreviations: EGF, epidermal growth factor; eNOS, endothelial nitric oxide synthase; HB-EGF, heparin-binding epidermal growth factor; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; TGF, transforming growth factor

Results

Expression of nNOS mRNA in human tissues and cells RT-PCR analyses for nNOS-specific mRNA species using different human tissues demonstrated that expression of the nNOS gene was not restricted to neuronal tissue (Fig 1). Amplicons represented processed nNOS transcripts, as the primers were located in exon 13 and exon 15 of the genomic sequence (Fig 1a). Interestingly, processed nNOS mRNA was also found in human skin biopsies and also human primary keratinocytes and the human keratinocyte cell line HaCaT (Fig 1b).

Differential expression of nNOS mRNA and protein during skin repair As we had detected the expression of nNOS mRNA in skin tissue, we now investigated the presence of nNOS mRNA and protein in a model of skin repair in mice. As tightly controlled epithelial tissue movements during skin regeneration were associated with an undisturbed expression of the inducible (Frank *et al*, 1999; Stallmeyer *et al*, 1999; Kämpfer *et al*, 2003) and endothelial (Stallmeyer *et al*, 2002) NOS isoenzymes, we determined expression patterns of the neuronal-type NOS isoform in normal skin and in skin after injury in mice. We isolated total RNA and protein from full-thickness excisional wounds at different intervals after injury and performed Real Time-PCR or immunoblots. Sixteen wounds (for RNA) and four wounds (for protein) from the backs of four mice were excised for each time point. They were combined and used for RNA or protein isolation. Normal skin from the back of non-wounded mice was used as a control. Non-wounded skin was characterized by a low-level expression of nNOS at the mRNA and protein level (Fig 2a and b); however, nNOS mRNA rapidly declined shortly after wounding (Fig 2a). As repair moved on, interestingly, we observed a temporal delay of the appearance of nNOS protein as compared with expression of its mRNA. Data revealed a dramatic induction
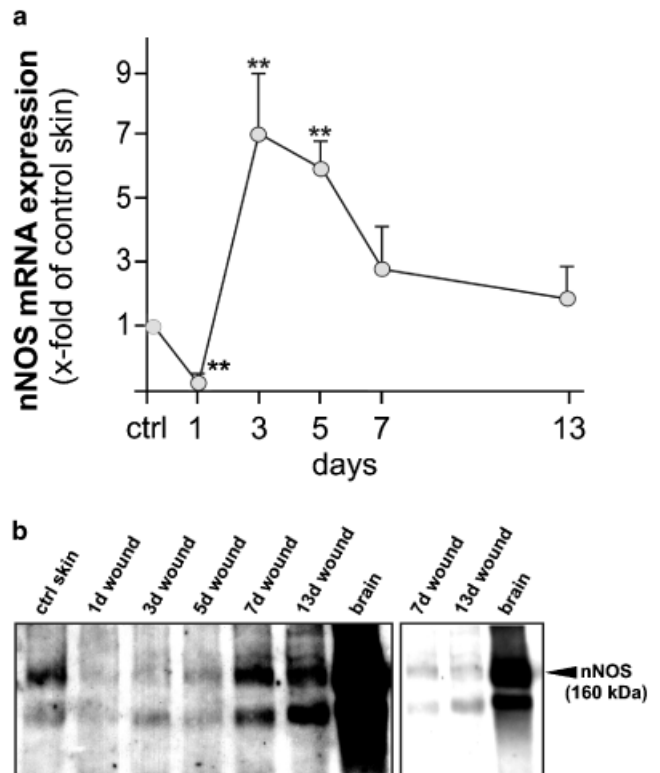
Figure 2
Expression of nNOS upon skin wounding. Regulation of nNOS mRNA (*a*) and protein (*b*) expression in BALB/c mice as assessed by real-time PCR or immunoblot. The time after injury is indicated. *Ctrl skin* represents back skin biopsies of non-wounded mice. A quantification of nNOS mRNA (x-fold induction compared with control skin) is shown in (*a*). ∗∗, p < 0.01 (ANOVA) compared with control skin. Bars represent mean ± SD obtained from wounds (n = 48) isolated from animals (n = 12) from three independent animal experiments. RNAs have been equilibrated by analysis of constitutive porphobilinogen deaminase. (*b*) Total protein (50 μg) from non-wounded control skin and wound tissue was analyzed for nNOS-specific protein. A lysate from murine brain tissue was used as to control the specificity of the used anti-nNOS antiserum. A shorter exposure time of the immunoblot is shown in the *right panel*. nNOS, neuronal nitric oxide synthase.

of nNOS mRNA at day 3 post-wounding (Fig 2a), which was not paralleled by a marked induction of nNOS protein. It is remarkable that decreasing levels of nNOS mRNA during later phases of repair (days 7–14) were directly correlated with a strong increase in nNOS protein (Fig 2a and b).

Localization of nNOS expression at the wound site Next, we directed our attention to immunolocalize the nNOS isoform at the wound site. To do so, sections from 1, 5, and 13 d full-thickness wounds were stained with a mono-specific, polyclonal antibody against murine nNOS protein. At all time points, nNOS immunostaining revealed similar expression pattern within the wound. Serial section from 5 d is presented in Fig 3. Interestingly, nNOS was localized at epithelial sites at the margins of the wound (Fig 3a). Moreover, we detected an accumulation of nNOS protein directly adjacent to the basal lamina (Fig 3a and b). Especially, the basal keratinocytes of the migrating epithelial were characterized by a marked expression of nNOS protein (Fig 3c and d); however, some strongly positive
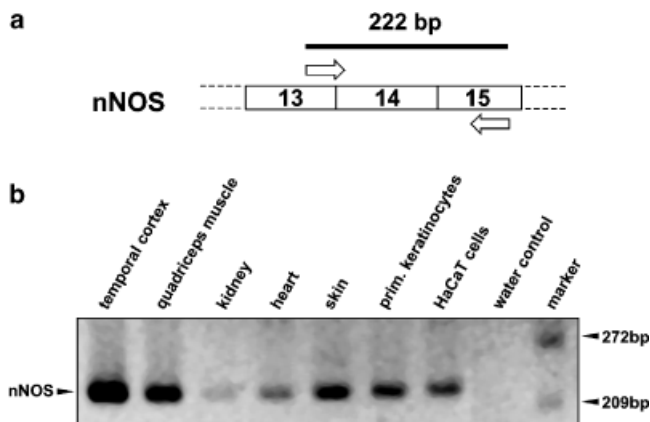
Figure 1
nNOS mRNA expression in human tissues and cells. (*a*) nNOS exonic organization (exons 13–15). Arrows show the localization of the sense and antisense primers used for PCR amplification. Exonic localization of primers indicate that PCR amplicons (222 bp) must represent expressed nNOS mRNA. (*b*) Amplifications of nNOS-specific sequences were performed using reverse-transcribed mRNA from human tissues and cells as indicated. nNOS, neuronal nitric oxide synthase.
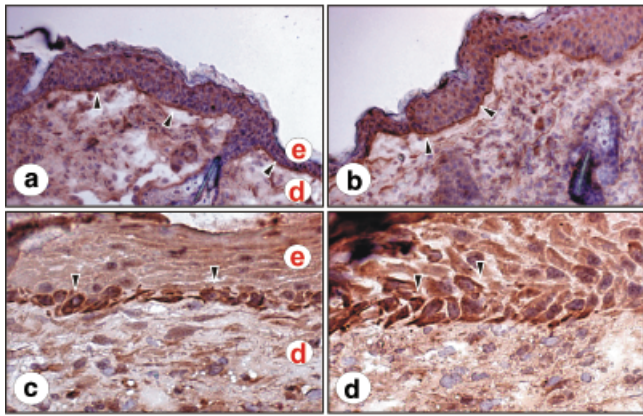
Figure 3
Localization of nNOS protein in regenerating skin. Frozen sections from mouse wounds (day 5 post-wounding) were incubated with a polyclonal antiserum directed against nNOS. Sections were subsequently stained with the avidin–biotin–peroxidase complex system using 3,3-diaminobenzidine tetrahydrochloride as a chromogenic substrate. Nuclei were counterstained with hematoxylin. Immunopositive signals within the sections are indicated with arrows. *d*, dermis; *e*, epidermis. nNOS, neuronal nitric oxide synthase.

cells with fibroblast-like morphology were localized within the underlying granulation tissue (Fig 3*c*).

nNOS regulation in human keratinocytes (HaCaT) As we had detected wound keratinocytes as a source of nNOS protein expression in skin repair, we now used the human keratinocyte cell line HaCaT as a model to determine possible regulators of nNOS expression *in vitro*. As shown in Fig 4*a*, keratinocytes revealed a basal expression of nNOS mRNA (Fig 4*a*, *left panel*). As a next step, we stimulated quiescent cells with a variety of growth factors and cytokines. Interestingly, none of the applied growth factors (PDGF, KGF, TGF-β1) or cytokines (IL-1β, TNF-α, IFN-γ) was able to enhance nNOS mRNA expression in the cells (data not shown). But treatment of cells with EGF resulted in a marked induction of nNOS mRNA levels (Fig 4*a*), which was specific for the neuronal isoform of the NOS isoenzymes (Fig 4*a*, *middle* and *right panels*). Stimulation (5-fold) of nNOS mRNA by EGF peaked after 12 h and, moreover, we detected significantly elevated levels of nNOS mRNA even after 24 h of treatment (Fig 4*b*). A dose–response experiment revealed that even low concentrations of EGF (1 ng per mL) stimulated nNOS expression in HaCaT keratinocytes (Fig 4*c*). Finally, we could confirm the EGF-mediated increase in nNOS mRNA expression at the protein level (Fig 4*d*). Densitometric analyses of four different blots from four independent cell culture experiments demonstrated an increase in nNOS protein of 270% ± 27% of control (mean ± SD).

Additional ligands of the ErbB network stimulate nNOS expression As the EGFR, and the ErbB2, ErbB3, and ErbB4 receptors constitute the EGFR family of protein tyrosine kinases, we determined the presence of ErbB2, ErbB3, and ErbB4 in human primary and also HaCaT keratinocytes using RT-PCR. With exception of human primary cells, which did not express ErbB4, we could detect

amplicons for all the other members of the EGFR family in both keratinocyte cell types (data not shown). As the multiplicity and redundancy of ligands for the EGFR family is well documented (Yarden and Sliwkowski, 2001), we speculated that additional ligands of the EGFR family might also serve as stimulators of nNOS expression in keratinocytes. To this end, we treated cultured HaCaT cells with heparin-binding EGF (HB-EGF), TGF-α, and two splice variants of the neuregulin gene, hereregulin-α, and hereregulin-β1. As shown in Fig 5, RNase protection assays clearly demonstrated an upregulation of nNOS mRNA with other ligands of the EGFR family.

Temporal and spatial correlation of activated EGFR and nNOS at the wound site As activation of the EGFR in keratinocytes increased the observed basal levels of nNOS mRNA in these cells *in vitro* (Figs 4 and 5), we hypothesized that we might find an activated EGFR during skin repair that might be associated with nNOS expression with respect to time and localization. Moreover, expression of the EGFR has been demonstrated to be present in wounded tissue (Stoscheck *et al*, 1992). First, we determined the kinetics of activated EGFR in wound tissue. To this end, we analyzed the immunoblots with a phospho-specific (Y-1173) antibody against the EGFR. Not unexpected, we found a transient increase in activated EGFR during the inflammatory and proliferative phase of repair (Fig 6*a*), clearly matching the observed induction of nNOS mRNA after wounding (Fig 2*a*). But elevated levels of nNOS protein at late stages of repair (Fig 2*b*) were clearly associated with declining amounts of active EGFR (Fig 6*a*). Additionally, immunohistochemistry from 5-d (Fig 6*b*) and 7-d (Fig 6*c*) wounds showed a partial co-localization of phosphorylated EGFR in wound keratinocytes as compared with nNOS (Fig 3).

EGFR-mediated signaling pathways targeting nNOS upregulation As a next step, we investigated a possible contribution of different components of the intracellular EGF-signaling network, that is known to originate from the membrane-bound EGFR, to nNOS expression. To this end, we treated HaCaT keratinocytes with EGF in the presence or absence of several inhibitors. Samples of total cellular RNA were subsequently analyzed by RNase protection assay. Not unexpected, we observed a dose-dependent inhibition of EGF-mediated nNOS expression in the presence of the specific EGFR antagonists PD153035 (Fig 7*a*) and the EGFR and Janus kinase (JAK)-2/3 inhibitor AG490 (Fig 7*b*). Unlike the PD153035 compound, we found that AG490 was also able to inhibit the basal expression of nNOS in the cells (Fig 7*a* and *b*). We also tested inhibitors of the mitogen-activated protein kinase (MAPK) pathways, but specific MEK1,2 inhibitors (U0126 and PD98059) as well as the inhibitor of the p38 MAPK (SB203580) had no effect on EGF-mediated nNOS expression (data not shown). Moreover, also inhibition (Gö6983) or activation (PDBu) of the phospholipase C/protein kinase C pathway, or inhibition of the PI3 kinase/protein kinase B pathway by wortmannin and LY294002 did not alter the EGF-induced stimulation of nNOS expression in HaCaT keratinocytes (data not shown).
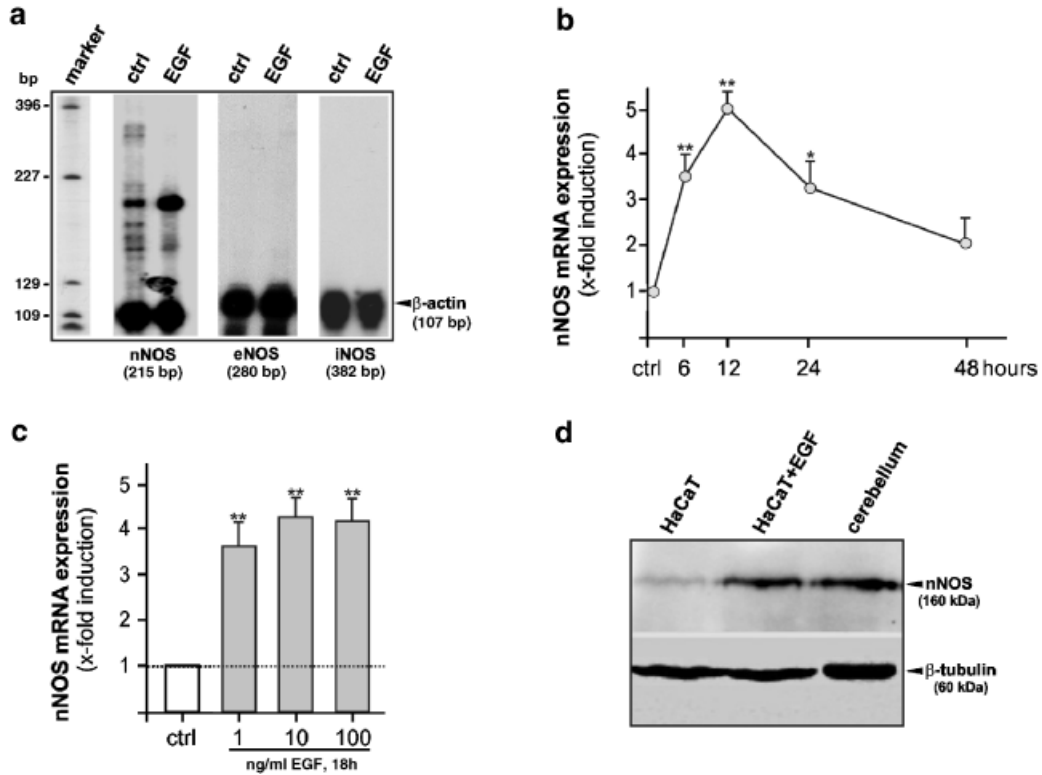
Figure 4
Human HaCaT keratinocytes increase nNOS expression upon EGF treatment. (*a*) Quiescent human HaCaT keratinocytes were stimulated with EGF (100 ng per mL) for 24 h. RNase protection assay to detect nNOS-, eNOS-, or iNOS-specific mRNA was performed as indicated. *Ctrl*, non-stimulated cells. One representative experiment is shown. β-actin expression is shown as a loading control within the same gel. A quantification of nNOS mRNA (x-fold induction compared with control) over a time period of 24 h as indicated is shown in (*b*). ∗, $p < 0.05$; ∗∗, $p < 0.01$ (ANOVA) compared with control. Bars indicate the mean ± SD obtained from four independent cell culture experiments. (*c*) Quiescent HaCaT keratinocytes were treated with increasing amounts of EGF for 18 h as indicated. A quantification of nNOS mRNA (x-fold induction compared with control) is shown. ∗∗, $p < 0.01$ (ANOVA) compared with control. Bars indicate the mean ± SD obtained from four independent cell culture experiments. (*d*) Total protein (100 μg) from quiescent and EGF-treated (100 ng per mL) HaCaT cells was analyzed for nNOS-specific protein by immunoblot as indicated. Lysate from porcine cerebellum was used to control specificity of the anti-nNOS antiserum. β-tubulin was used as a loading control. One representative experiment is shown. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; EGF, epidermal growth factor.
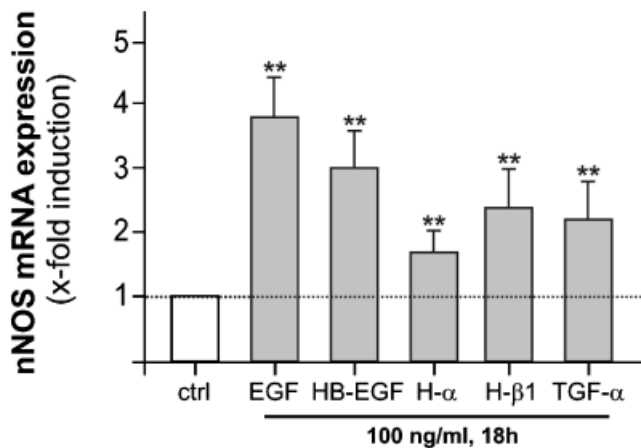

Figure 5
Upregulation of nNOS mRNA by ErbB receptor ligands in HaCaT cells. Quiescent human HaCaT keratinocytes were stimulated with EGF, heparin-binding EGF (HB-EGF), heregulin (H)-α, H-β1, or transforming growth factor-α (TGF-α) (100 ng per mL) for 18 h as indicated. RNase protection assay to detect nNOS-specific mRNA was performed. *Ctrl*, non-stimulated cells. A quantification of nNOS mRNA (x-fold induction compared with control) is shown. ∗∗, $p < 0.01$ (ANOVA) compared with control. Bars indicate the mean ± SD obtained from four independent cell culture experiments. nNOS, neuronal nitric oxide synthase.

Discussion

Wound healing comprises a highly coordinated process that leads to an at least partial reconstruction of the injured tissue. Evidently, recent studies suggested an important role of the short-lived mediator NO in skin repair (Frank *et al*, 2002). NO is synthesized from L-arginine by NOS, which exists in three different isoforms (Moncada *et al*, 1991; Alderton *et al*, 2001). As L-arginine represents the only substrate for NOS activity, this enzymatic reaction links the long-known observation that L-arginine was implicated in wound healing (Seifter *et al*, 1978) with the potency of NO to drive cutaneous repair. Although eNOS has been shown expressed in wound margin keratinocytes upon wounding (Stallmeyer *et al*, 2002), recent data from iNOS-deficient mice identified iNOS-derived NO as a pivotal player in wound healing (Yamasaki *et al*, 1998). In line, inhibition of iNOS enzymatic activity during repair using N6- (imi-noethyl)-L-lysine evidenced a potent effect of iNOS-derived NO on epithelial gene expression leading to severely impaired re-epithelialization movements (Frank *et al*, 1999, 2000; Stallmeyer *et al*, 1999). Moreover, recent data from our laboratory showed that downregulation of iNOS as well as eNOS, was clearly associated with impaired healing
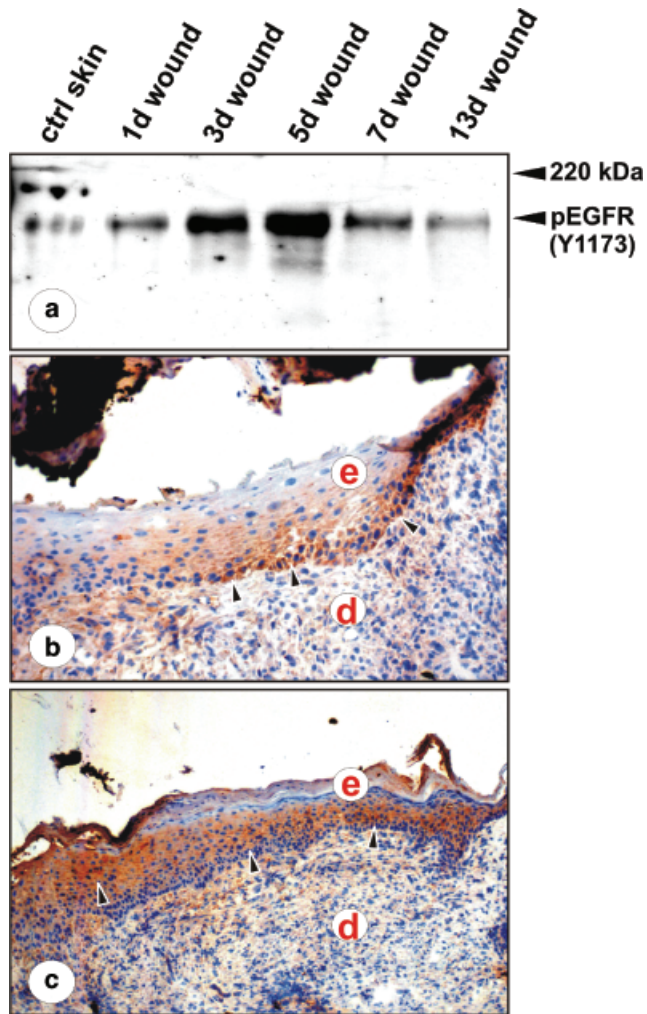
Figure 6
Expression of activated epidermal growth factor receptor (EGFR) at the wound site. (*a*) Total protein (50 μg) from non-wounded (*ctrl skin*) and wounded skin was analyzed for the presence of phosphorylated (Y1173) EGFR-specific protein by immunoblot as indicated. One representative experiment is shown. Frozen sections from mouse wounds isolated at day 5 (*b*) and day 7 (*c*) post-wounding were incubated with a polyclonal antiserum directed against phosphorylated (Y1173) EGFR. Sections were subsequently stained with the avidin–biotin–peroxidase complex system using 3,3-diaminobenzidine tetra-hydrochloride as a chromogenic substrate. Nuclei were counterstained with hematoxylin. Immunopositive signals within the sections are indicated with arrows. *d*, dermis; *e*, epidermis.

Figure 7
EGFR antagonists inhibit nNOS mRNA expression in HaCaT cells. Quiescent HaCaT keratinocytes were treated with EGF (100 ng per mL) in the presence or absence of increasing amounts of the EGFR antagonist PD153035 (*a*) or the EGFR/JAK2/3 inhibitor AG490 (*b*) for 24 h as indicated. RNase protection assay to detect nNOS-specific mRNA was performed. A quantification of nNOS mRNA (x-fold induction compared with control) is shown. $**$, $p < 0.01$ (ANOVA) compared with control. Bars indicate the mean $\pm$ SD obtained from four independent cell culture experiments ($n = 4$). EGFR, epidermal growth factor receptor; nNOS, neuronal nitric oxide synthase.

conditions in diabetic mice (Stallmeyer *et al*, 2002; Kämpfer *et al*, 2003). To close the gap of non-available data concerning a potential presence of the third NOS isoform in skin repair, we focussed on nNOS expression in skin tissue.

First, our data confirmed previous data that had shown the presence of nNOS in skin-derived cell types such as keratinocytes and melanocytes (Baudouin *et al*, 1996; Romero-Graillet *et al*, 1996; Ormerod *et al*, 1998). In line with these findings, we could find a constitutively expressed nNOS in normal skin of mice. Moreover, we detected a particularly strong expression of nNOS in wound margin keratinocytes after wounding. Interestingly, nNOS protein expression peaked in late stages of repair, although its mRNA was induced more rapidly. This finding strongly argues for a stable nNOS mRNA species that is character-ized by a long mRNA half-life even in dynamic processes
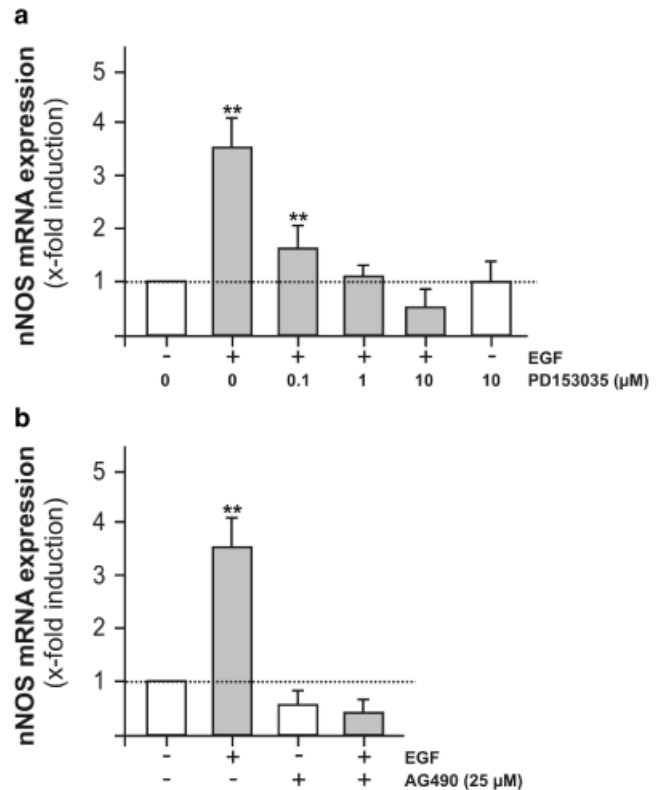
such as wound healing. Förstermann *et al* (1998) showed that constitutive expression of nNOS is associated with a long half-life of its mRNA. In contrast, short message half-life was observed for the inducible isoform. This finding is further supported by the considerable stability of nNOS mRNA in HaCaT cells shown in this study. From our data, it is reasonable to suggest a delayed translation of stable nNOS mRNA molecules that might be induced by mediators during the inflammatory phase of repair, into protein during late repair. Interestingly, cell culture experiments demon-strated that induction of nNOS was restricted to an activation of the EGFR; however, especially the potent EGFR ligands EGF and TGF-α are well known to be pre-sent during the inflammatory phase of repair (Martin, 1997; Singer and Clark, 1999). Interestingly, published data demonstrated that the iNOS isoform is expressed during wound inflammation and represents the predominant source of NO that finally drives tissue regeneration (Frank *et al*, 1998a, 1999; Yamasaki *et al*, 1998). By contrast, nNOS protein levels were markedly increased when wound inflammation had declined, suggesting a potential role for nNOS-derived NO in keratinocyte proliferation rather than inflammation. Evidently, here we describe an additional situation where an initially considered 'constitutively' ex-pressed gene is indeed regulated (Förstermann *et al*, 1998).

EGF represents a potent mitogen for epithelial cells and EGF is known to contribute to re-epithelialization processes during wound healing (Nanney, 1990). Moreover, expression of the EGFR is elevated in repair (Stoscheck *et al*, 1992). Here we found a transient increase in activated EGFR during the inflammatory and proliferative phase of repair, clearly matching the observed induction of nNOS mRNA after wounding. Interestingly, from all mediators tested, only ligands of the EGFR family were able to increase basal nNOS expression in HaCaT keratinocytes. More important, EGF stimulation was specific for nNOS, as eNOS and iNOS messages were not detectable in basal or EGF-induced conditions. Nevertheless, at least iNOS could be strongly induced by cytokines in the cells (Frank *et al*, 1998b). EGFR is able to heterodimerize with three additional members of the EGFR familiy (ErbB). In combination with ErbB2–ErbB4, receptor heterodimerization allows signaling from additional ligands such as HB-EGF, heregulin-α or heregulin-β1 (Yarden and Sliwkowski, 2001). In line, a formation of homo- and heterodimers between the ErbB tyrosine kinases has been demonstrated in HaCaT cells (Marques *et al*, 1999). Interestingly, the PDZ domain of ErbB4 interacts with a complex containing nNOS (Garcia *et al*, 2000; Huang *et al*, 2000). Heregulin-β1 was shown to upregulate nNOS expression (Krainock and Murphy, 2001b) in neuronal cells; however, in contrast to keratinocytes, upregulation of nNOS in neurons by heregulin-β1 was dependent on activation of the MAPK pathway in the cells (Krainock and Murphy, 2001a), indicating that activation of different signaling routes from ErbB receptors might be cell-type specific.

Additional data demonstrated that EGF did not stimulate the respective nNOS promoter activity, but increased the stability of nNOS mRNA (data not shown).

In summary, our study suggests that nNOS is constitutively expressed in normal skin tissue, and induced in late phases of skin repair. Keratinoyctes were the predominant nNOS expressing cell type at the wound site, and *in vitro* experiments revealed EGF as a potent inducer of nNOS expression in the cells. Thus, it is reasonable to suggest that, besides the iNOS and eNOS isoforms, also nNOS enzymatic activity might contribute to a functional NO production in wound tissue.

Materials and Methods

Wounding and preparation of wound tissues BALB/c mice were obtained from Charles River (Sulzfeld, Germany) and maintained under a 12-h-light/12-h-dark cycle at 22°C until they were 8 wk of age. At this time they were caged individually and wounded as previously described (Frank *et al*, 1999). Briefly, six full-thickness wounds were created on each animal, and skin biopsy specimens were taken 1, 3, 5, 7, and 14 d after injury. For every experimental time point, the wounds from four animals and the non-wounded back skin from four animals were combined and used for RNA and protein isolation, respectively. All animal experiments were carried out according to the guidelines and with the permission of the local government of Hessen, Germany.

Cell culture The human keratinocyte cell line HaCaT (Boukamp *et al*, 1988) was kindly provided by Dr N. E. Fusenig, German Cancer Research Center (DKFZ), Heidelberg, Germany. HaCaT cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FCS), 2 mmol per liter L-glutamine,

and 1% penicillin/streptomycin. Under these culture conditions, the HaCaT cells remain proliferative and undifferentiated. Before treatment with various EGFR ligands (f.c. 100 ng per mL), the cells were grown to confluency without changing the medium and rendered quiescent by a 24 h incubation in serum-free DMEM. Human primary epidermal keratinocytes were purchased from BioWhittaker Europe Inc. (Verviers, Belgium) and cultured in a defined keratinocyte medium (KBM-2) according to the instructions of the supplier. All cells were maintained at 37°C in 10% CO_2. DMEM, FCS and cell culture reagents were from Invitrogen/GIBCO (Groningen, The Netherlands).

Reverse transcription- (RT-) PCR and RT-real time-PCR Reverse transcription (RT) was performed using 2 μg total RNA purified from various human tissues, primary keratinocytes and HaCaT cells as template, a combination of oligo(dT)$_{12-18}$ and random hexamers as primers and Superscript reverse transcriptase (Invitrogen, Groningen, The Netherlands). The obtained cDNAs served as templates in PCR using Expand polymerase (Roche Diagnostics, Mannheim, Germany). For detection of nNOS expression, the oligonucleotides: 5′-CTT CAA GAA GCT AGC AGA AGC TGT-3′ and 5′-ACA AGG ACC AGA GTT TCA TGT TC-3′ were used as sense and antisense primers, respectively. Sequencing of the 222 nucleotide PCR product demonstrated the amplification of nNOS mRNA (end of exon 13, exon 14, part of exon 15, EMBL Acc. No. NM_000620). For the analysis of the various EGFR receptors subtypes, the following primer pairs were used: 5′-ACT CCT TCA CAC ATA CTC CTC CTC-3′ and 5′-CAT CTC CAT CAC TTA TCT CCT TGA-3′ (for EGFR, EMBL Acc. No. XXX), 5′-GAC ATT GAC GAG ACA GAG TAC CAT-3′ and 5′-CAT GTA GAC ATC AAT GGT GCA GAT-3′ (for ErbB2, EMBL Acc. No. X03363), 5′-ACC AGA CAC TGT ACA AGC TCT ACG-3′ and 5′-GCT GGA GTT GGT GTT ATA GTT CAA-3′ (for ErbB3, EMBL Acc. No. M34309) or 5′-GCA AAG TAC ACA TAT GGA GCA TTC-3′ and 5′-ATT CCC ATT GAT CTT GGT ACA GTT-3′ (for ErbB4, EMBL Acc. No. L07868).

nNOS message in non-wounded and wounded skin of mice was quantified by real time (RT) PCR using the iCyler (BioRad, Munich, Germany). One step RT-PCR was performed on 500 ng of total RNA isolated from biopsies using the QuantiTect Probe RT-PCR mix (Qiagen, Milden, Germany). For detection of murine nNOS expression, the oligonucleotides 5′-GGT GGA GAT TAA CAT TGC TGT CC TA-3′ and 5′-TTC TCC ATG TGT TTG ATG AAG GAC T-3′ were used as primers, and 5′-6FAM-ACA GCT TCC AGA GTG ACA AGG TGA CCA TTG-TAMRA served as the fluorescent TaqMan probe. Levels of nNOS were normalized by amplification of the murine constitutive porphobilinogen deaminase (PBGD) mRNA (5′-CCC TGG CAT ACA GTT TGA AAT CA-3′ and 5′-AGG AGG TAG TAT GGT AGG CAC ATC C-3′ as primers and 5′-6 JOE-ACC AAG GAG CTA GAA AAC GCC CTG GA-TAMRA-3′ as Taqman probe). Fluorescence was monitored at each cycle during the annealing/extension step.

RNA isolation and ribonuclease protection assays Total cellular RNA was isolated as described previously (Chomczynski and Sacchi, 1987). The plasmids containing cDNAs for human nNOS (Boissel *et al*, 2003), for human eNOS (Kleinert *et al*, 1998), for iNOS (Kleinert *et al*, 1996), and for β-actin (Gräf *et al*, 2001) have been described previously. Expression levels of different mRNAs species were determined from total cellular RNA by RNase protection assay as described (Kleinert *et al*, 1998; Wallerath *et al*, 1999). Briefly, an antisense transcript was synthesized *in vitro* using T3 or T7 RNA polymerase and [α-^{32}P]UTP (800 Ci per mmol). RNA samples were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% acrylamide/8 M urea gels and analyzed using a Phospho-Imager (BioRad, Munich, Germany). Values of protected bands were normalized using the corresponding β-actin bands. RNases A and T1 were from Roche Diagnostics.

Histological analysis Mice were wounded as described above. Animals were euthanized at days 5 and 7 after injury. Complete wounds were isolated from the back, bisected, and frozen in tissue-freezing medium. Six-micrometer frozen sections were fixed with acetone and treated for 10 min at room temperature with 1% H_2O_2 in PBS to inactivate endogenous peroxidases. Sections were subsequently incubated for 60 min at room temperature with a rabbit polyclonal antiserum recognizing the murine nNOS protein and tyrosine-phosphorylated EGFR (Y1173) (Santa Cruz Biotechnology Inc., Heidelberg, Germany).

Preparation of protein lysates and western blot analysis Skin and cell culture samples were homogenized in lysis buffer (1% Triton X-100, 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM ethylendiaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 15 μg per mL leupeptin). The extracts were cleared by centrifugation (Kämpfer *et al*, 1999; Stallmeyer *et al*, 1999). Fifty micrograms of total protein from skin or cellular lysates was separated using SDS-gel electrophoresis. After transfer to a polyvinylidene difluoride (PVDF) membrane, murine nNOS and tyrosine (Y1173)-phosphorylated EGFR protein were detected using the polyclonal antisera (Santa Cruz Biotechnology Inc., Heidelberg, Germany). To assure equal loading, Ponceau red staining was performed. For detection of human nNOS protein in HaCaT keratinocyte lysates, a mouse monoclonal antibody was used (BD Transduction Laboratories, Heidelberg, Germany). As a loading control, blots were probed with a mouse monoclonal antibody against β-tubulin (Sigma, Deisenhofen, Germany). A secondary antibody coupled to horseradish peroxidase and the ECL detection system were used to visualize the nNOS and phosphorylated EGFR proteins, respectively. Phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were from Sigma (Deisenhofen, Germany) or Roche Diagnostics (Mannheim, Germany). The ECL detection system was obtained from Amersham (Freiburg, Germany).

Statistics Data are shown as means ± SD. Data analysis was carried out using the unpaired Student's *t* test with raw data. Statistical comparison between more than two groups was carried out by ANOVA and Fisher's protected least-significant-difference (PLSD) test.

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References

Alderton WK, Cooper CE, Knowles RG: Nitric oxide synthases structure function inhibition. Biochem J 357:593–615, 2001

Baudouin JE, Tachon P: Constitutive nitric oxide synthase is present in normal human keratinocytes. J Invest Dermatol 106:428–431, 1996

Boissel JP, Zelenka M, Gödtel-Armbrust U, Feuerstein TJ, Förstermann U: Transcription of different exons 1 of the human neuronal nitric oxide synthase gene is dynamically regulated in a cell- and stimulus-specific manner. Biol Chem 384:351–362, 2003

Boukamp P, Petrussevska RT, Breitkreuz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771, 1988

Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V: Nitric oxide and its implications in skin homeostasis and disease—A review. Arch Dermatol Res 290:643–651, 1998a

Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V: Nitric oxide in human skin: Current status and future prospects. J Invest Dermatol 110:1–7, 1998b

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159, 1987

Dawson VL, Dawson TM: Nitric oxide in neurodegeneration. Prog Brain Res 118:215–229, 1998

Förstermann U, Boissel J P, Kleinert H: Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J 12:773–790, 1998

Förstermann U, Closs EI, Pollock J S, Nakane M, Schwarz P, Gath I, Kleinert H: Nitric oxide synthase isozymes, characterization, purification, molecular cloning, and functions. Hypertension 23:1121–1131, 1994

Frank S, Kolb N, Werner ER, Pfeilschifter J: Coordinated induction of inducible nitric oxide synthase and GTP-cyclohydrolase I is dependent on inflammatory cytokines and interferon-gamma in HaCaT keratinocytes: Implications for the model of cutaneous wound repair. J Invest Dermatol 111:1065–1071, 1998b

Frank S, Kämpfer H, Wetzler C, Pfeilschifter J: Nitric oxide drives skin repair: Novel functions of an established mediator. Kidney Int 61:882–888, 2002

Frank S, Kämpfer H, Wetzler C, Stallmeyer B, Pfeilschifter J: Large induction of the chemotactic cytokine RANTES during cutaneous wound repair: A regulatory role for nitric oxide in keratinocyte-derived RANTES expression. Biochem J 347:265–273, 2000

Frank S, Madlener M, Pfeilschifter J, Werner S: Induction of inducible nitric oxide synthase and its corresponding tetrahydrobiopterin-cofactor-synthesizing enzyme GTP-cyclohydrolase I during cutaneous wound repair. J Invest Dermatol 111:1058–1064, 1998a

Frank S, Stallmeyer B, Kämpfer H, Kolb N, Pfeilschifter J: Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair. FASEB J 13:2002–2014, 1999

Garcia RA, Vasudevan K, Buonanno A: The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. Proc Natl Acad Sci USA 97:3596–3601, 2000

Gräf P, Förstermann U, Closs EI: The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C. Br J Pharmacol 132:1193–1200, 2001

Huang YZ, Won S, Ali DW, et al: Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. Neuron 26:443–455, 2000

Kämpfer H, Kahlina U, Mühl H, Pfeilschifter J, Frank S: Counterregulation of interleukin-18 mRNA and protein expression during cutaneous wound repair in mice. J Invest Dermatol 113:369–374, 1999

Kämpfer H, Pfeilschifter J, Frank S: Expression and activity of arginase isoenzymes during normal and diabetes-impaired skin repair. J Invest Dermatol 121:1544–1551, 2003

Kleinert H, Euchenhofer C, Ihrig-Biedert I, Förstermann U: Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-kappa B. Mol Pharmacol 49:15–21, 1996

Kleinert H, Wallerath T, Euchenhofer C, Ihrig Biedert I, Li H, Förstermann U: Estrogens increase transcription of the human endothelial NO synthase gene: Analysis of the transcription factors involved. Hypertension 31:582–588, 1998

Krainock R, Murphy S: Regulation of functional nitric oxide synthase-1 expression in cerebellar granule neurons by heregulin is post-transcriptional, and involves mitogen-activated protein kinase. J Neurochem 78:552–559, 2001a

Krainock R, Murphy S: Heregulin upregulates the expression of nitric oxide synthase (NOS)-1 in rat cerebellar granule neurons via the ErbB4 receptor. J Neurochem 76:312–315, 2001b

Marques MM, Martinez N, Rodriguez-Garcia I, Alonso A: EGFR family-mediated signal transduction in the human keratinocyte cell line HaCaT. Exp Cell Res 252:432–438, 1999

Martin P: Wound healing—Aiming for perfect skin regeneration. Science 276: 75–81, 1997

Moncada S, Palmer RM, Higgs EA: Nitric oxide, physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–142, 1991

Nanney LB: Epidermal and dermal effects of epidermal growth factor during wound repair. J Invest Dermatol 94:624–629, 1990

Ormerod AD, Weller R, Copeland P, Benjamin N, Ralston SH, Grabowksi P, Herriot R: Detection of nitric oxide and nitric oxide synthases in psoriasis. Arch Dermatol Res 290:3–8, 1998

Romero-Graillet C, Aberdam E, Biagoli N, Massabni W, Ortonne JP, Ballotti R: Ultraviolet B radiation acts through the nitric oxide and cGMP signal transduction pathway to stimulate melanogenesis in human melanocytes. J Biol Chem 271:28052–28056, 1996

Romero-Graillet C, Aberdam E, Clement M, Ortonne JP, Ballotti R: Nitric oxide produced by ultraviolet-irradiated keratinocytes stimulates melanogenesis. J Clin Invest 99:635–642, 1997

Saur D, Seidler B, Paehge H, Schusdziarra V, Allescher HD: Complex regulation of human neuronal nitric-oxide synthase exon 1c gene transcription. Essential role of Sp and ZNF family members of transcription factors. J Biol Chem 277:25798–25814, 2002

Seifter E, Rettura G, Barbul A, Levenson SM: Arginine: An essential amino acid for injured rats. Surgery 84:224–230, 1978

Shimizu Y, Sakai M, Umemura Y, Ueda H: Immunohistochemical localization of nitric oxide synthase in normal human skin: Expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. J Dermatol 24:80–87, 1997

Singer AK, Clark RAF: Cutaneous wound healing. N Engl J Med 341:738–746, 1999

Stallmeyer B, Anhold M, Wetzler C, Kahlina K, Pfeilschifter J, Frank S: Regulation of eNOS in normal and diabetes-impaired skin repair: Implications for tissue regeneration. Nitric Oxide 6:168–177, 2002

Stallmeyer B, Kämpfer H, Kolb N, Pfeilschifter J, Frank S: The function of nitric oxide wound repair: Inhibition of inducible nitric oxide synthase severely impairs wound reepithelialization. J Invest Dermatol 113:1090–1098, 1999

Stoscheck CM, Nanney LB, King LE Jr: Quantitative determination of EGF-R during epidermal wound healing. J Invest Dermatol 99:645–649, 1992

Wallerath T, Witte K, Schäfer SC, et al: Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. Proc Natl Acad Sci USA 96:13357–13362, 1999

Wang Y, Newton DC, Robb GB, et al: RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. Proc Natl Acad Sci USA 96:12150–12155, 1999

Weller R: Nitric oxide, skin growth and differentiatio: More questions than answers? Clin Exp Dermatol 24:388–391, 1999

Yamasaki K, Edington HD, McClosky C, et al: Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. J Clin Invest 101:967–971, 1998

Yarden Y, Sliwkowski MX: Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2:127–137, 2001