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Review

Rho GTPases and Nox dependent ROS production in skin. Is there a connection?

Alanna Stanley[1], Ailish Hynes[2], Cord Brakebusch[3] and Fabio Quondamatteo[1]

[1]Anatomy, National University of Ireland Galway, University Road, Galway, Ireland, [2]Physiology, National University of Ireland Galway, University Road, Galway, Ireland and [3]Biomedical Institute, BRIC, University of Copenhagen, Copenhagen, Denmark

Summary. Rho GTPases are a family of small GTP binding proteins most commonly known for the regulation of many cellular processes, including actin cytoskeleton re-organisation, cell proliferation, signal transduction and regulation of apoptosis. Additionally, a link between Rho GTPases and reactive oxygen species (ROS) has been shown. In line with the growing interest in the role of ROS in cell biology, the relevance of this connection is becoming increasingly clearer. ROS production is classically associated with oxidative metabolic pathways (e.g. respiratory chain, arachidonic acid). During these metabolic pathways, ROS are produced as by-products and these can be potentially toxic. However, numerous cell types contain dedicated enzymatic complexes, i.e., NADPH oxidase (Nox) complexes, for regulated production of ROS. This regulated production of ROS seems to be important for a number of fundamental cell biological processes, including cell growth, differentiation, migration, angiogenesis, aimed at maintaining tissue homeostasis. Data suggests that skin cells are capable of a regulated ROS production via Nox complexes. Members of the Rho GTPase family have been found to play a central regulatory role in Nox activity. In the present review we will focus on the involvement of Rho GTPases in regulated production of ROS with special emphasis on the skin. We will also discuss the possibility that some *in vivo* effects of the deletion of members of the Rho GTPase family in skin cells could potentially be linked to a reduced ability of regulated ROS production.

Key words: ROS, Nox complexes, Rho GTPases, Keratinocytes, Fibroblats

Offprint requests to: Dr. Fabio Quondamatteo, Anatomy, NUI Galway, University Road, Galway, Ireland. e-mail: fabio.quondamatteo@nuigalway.ie

Introduction

The skin is the organ of the body that is in constant contact with the external environment. Therefore, one of the most important functions of the skin is to act as a barrier and provide protection from external assaults such as UV radiation, pathogens, and pollutants. The maintenance of homeostasis in the skin requires the interaction of a number of signaling molecules controlling many cellular processes. A particularly interesting group of signaling molecules are reactive oxygen species (ROS), which at low levels regulate cellular processes including signaling, proliferation, migration, gene expression, immunity, and, wound healing (Quinn et al., 2006; Bedard and Krause, 2007). Conversely, ROS at higher concentrations can cause tissue damage and cell death (Lambeth, 2004; Rada and Leto, 2008).

The generation of ROS is complex, since they can be generated as by-products of oxygen metabolism (Lambeth, 2004) but also by dedicated enzymatic complexes, i.e., NADPH oxidase (Nox) complexes in a regulated manner (Lambeth, 2004; Geiszt, 2006). Among the factors that control expression and activity of Nox complexes, Rac proteins play a central role.

In this review, we now try to compile the current knowledge on how Rac proteins and other Rho GTPases might affect ROS production in skin and how this could potentially contribute to regulate keratinocyte and fibroblast functions in the skin *in vivo*.

Rho GTPase and their function in skin

Rho GTPases form one of the five families of the Ras Superfamily. The Rho GTPase family of (20-30 KDa) monomeric GTP binding proteins is made up of more than 20 members divided into 8 subfamilies,

namely Rho, Rac, Cdc42, Rnd, RhoD, RhoH/TTF, Rho BTB, and Miro (Table 1). In response to extracellular signals, they can act as molecular switches by cycling between an inactive GDP-bound and an active GTP-bound state, a process commonly referred to as the GTPase cycle (Fig. 1) (Bishop and Hall, 2000; Jaffe and Hall, 2005). In their active state, Rho GTPases can function as signalling molecules by binding and activating specific effector proteins. This subsequently results in downstream responses in the cell which include regulation and organisation of the actin cytoskeleton, transcriptional regulation, and number of morphogenetic signals (Takai et al., 2001; Karlsson et al., 2009).

The GTPase cycle involves a two-step process; GDP disassociation and GTP hydrolysis, which are tightly regulated by two families of intracellular proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) respectively (Bos et al., 2007). In response to extracellular signals, GEFs can bind to inactive Rho GTPase at the cell membrane, and cause a conformational change in their nucleotide binding site (Bos et al., 2007). This destabilises the nucleotide binding site resulting in the release of GDP leaving the nucleotide binding site free. Here, binding of GTP occurs due to its higher concentration in the cell (compared to that of GDP). Binding of GTP causes release of the GEF and finally activation of the Rho GTPase (Bos et al., 2007; Csepanyi-Komi et al., 2011). GAP proteins specific for Rho GTPases, function in their inactivation by increasing their rate of GTP hydrolysis (Bos et al., 2007; Csepanyi-Komi et al., 2011). GAPs can be regulated by a number of different mechanisms, including protein-protein interactions, phosphorylation and proteolytic degradation (Bernards and Settleman, 2004). GAP proteins specific for Rho GTPases contain a RhoGAP domain that interacts with the nucleotide binding domain. This causes a conformational change in the binding domain resulting in a decrease in the activation energy for hydrolysis, as well as changing the orientation of the bound GTP for better nucleophilic attack by water and final GTP hydrolysis (Bos et al., 2007; Csepanyi-Komi et al., 2011). This leaves the Rho GTPases bound to GDP, i.e. in an inactive state (Bishop and Hall, 2000; Jaffe and Hall, 2005)

Rho GTPase proteins are further regulated by guanine nucleotide dissociation inhibitors (GDIs) (Garcia-Mata et al., 2011). These factors can bind to both the GDP and the GTP bound forms of Rho GTPases and prevent nucleotide release, as well as possibly function in the localisation of the Rho GTPases to membranes. The main function of GDIs is however to maintain a stable cytosolic soluble pool of inactive Rho GTPase proteins by sequestering GDP-bound GTPases (Garcia-Mata et al., 2011). Due to the presence of a hydrophobic isoprenoid moiety, inactive Rho GTPases are degraded by water in the cytosol. In order to prevent their degradation, GDIs function in the removal of the inactive Rho GTPase from membranes, and, once bound, they shield the isoprenoid moiety. Conversely, by binding to the GTP bound form of Rho GTPases, GDIs prevent both intrinsic and GAP stimulated GTP hydrolysis, thus maintaining the Rho GTPases in their active state. GDIs are regulated by a number of different mechanisms including; protein-protein and protein-lipid interactions, and phosphorylation (Garcia-Mata et al., 2011).

Table 1. Rho GTPase subfamilies and members.

Rho GTPase Subfamily	Members of Subfamily
Rho	RhoA, RhoB and RhoC
Rac	Rac1, Rac2, Rac3 and RhoG
Cdc42	Cdc42, TC10, TCL, Chip, Wrch-1
Rnd	Rnd1, Rnd2, Rnd3/RhoE
RhoD	RhoD, Rif
RhoH/TTF	RhoH/TTF
RhoBTB	RhoBTB1 and RhoBTB2
Miro	Miro1 and Miro2

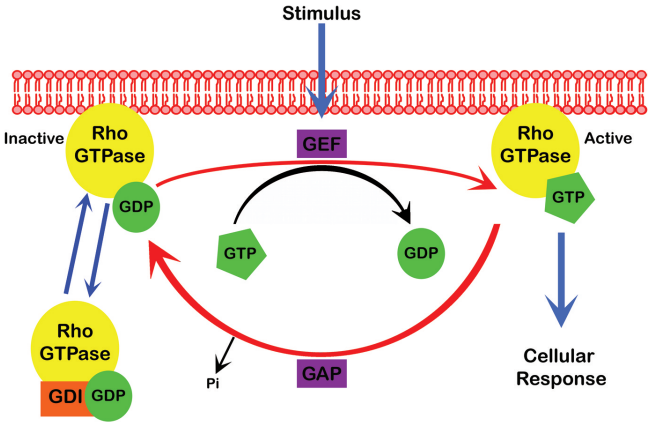

Fig. 1. The GTPase Cycle. (Bishop and Hall, 2000; Bernards and Settleman, 2004; Jaffe and Hall, 2005; Bos et al., 2007; Csepanyi-Komi et al., 2011) The GTPase cycle involves a two-step process; GDP disassociation and GTP hydrolysis, processes tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) respectively (purple). In response to extracellular signals, GEFs become activated and bind to the inactive form of a Rho GTPase (yellow) at the cell membrane. This causes a conformational change in the nucleotide binding site of the Rho GTPase resulting in the release of GDP (green circle). GEFs do not prevent nucleotide binding; therefore, binding of GTP (green pentagon) occurs due to its higher concentration in the cell, compared to that of GDP. Binding of GTP causes release of the GEF and activation of the Rho GTPase. GAP proteins for Rho GTPases contain a RhoGAP domain that interacts with the nucleotide binding domain of the Rho GTPase. When bound, the GAP protein causes a conformational change in the nucleotide binding domain of the Rho GTPase which ultimately results in an increase in the rate of GTP hydrolysis. This leads to release of phosphate (Pi) and formation of GDP which remains linked to the Rho GTPase, thus bringing the Rho GTPase into the inactive state. Rho GTPase proteins are further regulated by guanine nucleotide dissociation inhibitors (GDIs, orange). These can bind to either the inactive or active form, prevent nucleotide release, and maintain a stable cytosolic soluble pool of Rho GTPase proteins. Drawn modified according to Jaffe and Hall (2005).

Within the past six years considerable knowledge on the function of Rho GTPases in the skin *in vivo* has been generated by analysis of mice deficient for members of the Rho GTPase family (Benitah et al., 2005; Chrostek et al., 2006; Wu et al., 2006a,b; Benitah and Watt, 2007; Castilho et al., 2007; Tscharntke et al., 2007; Liu et al., 2008, 2009; Wang et al., 2010; Jackson et al., 2011).

Rac1 deficiency in keratinocytes has been reported to cause severe alterations and loss of hair follicles in two independent strains of mice following deletion of the Rac1 gene (Chrostek et al., 2006; Castilho et al., 2007). In a third KO-mouse line, extensive defects were observed in the interfollicular epidermis, which were suggested to be caused by a loss of keratinocyte stem cells (Benitah et al., 2005; Benitah and Watt, 2007).

The loss of the distorted Rac1-null hair follicles is preceded by an infiltration of macrophages in the skin of Rac1 KO mice, which removed the non-permanent part of the hair follicles (Chrostek et al., 2006). Also, hypopigmentation of the skin, abnormal expression of filaggrin and increased proliferation of the basal keratinocytes have been reported in absence of Rac1 in keratinocytes (Castilho et al., 2007).

Transgenic mice expressing the Rac1 dominant inhibitory mutant N17Rac1 in basal keratinocytes driven by keratin-14 showed no defects in the hair follicle maintenance, probably due to insufficient inhibition of Rac1 (Tscharntke et al., 2007).

No alterations in a number of signaling pathways analysed, namely JNK, p38, Akt, NF-kappaB and ERK, was found under physiological conditions, e.g. non-wounded or non-neoplastic skin (Chrostek et al., 2006; Wang et al., 2010). In contrast to this, in hyper-proliferative conditions, ERK phosphorylation was shown to be dependent on Rac1 both in Rac-null and dnRac1 expressing mice (Tscharntke et al., 2007; Wang et al., 2010). Both in wound healing and in a chemically induced skin tumour model, decreased phosphorylation of ERK1/2 was found in the absence of Rac1 (Tscharntke et al., 2007; Wang et al., 2010). This indicates that Rac1 is required for proper activation of the ERK signaling pathway involved in keratinocyte hyperproliferation.

In contrast to Rac1, the amount of published work on the deletion of other Rho GTPases in keratinocytes is more limited and is indeed restricted to RhoA and Cdc42 (Wu et al., 2006a,b; Jackson et al., 2011).

RhoA deficiency in keratinocytes had no major effect on the development and maintenance of the interfollicular epidermis (Jackson et al., 2011). However, it resulted in a significant reduction in the phosphorylation of myosin light chain and cofilin (Jackson et al., 2011). Both these proteins can by phosphorylated by Rho-associated protein kinase (ROCK), a downstream effector of Rho proteins (Matsui et al., 1996). Whereas *in vivo* levels of activated Rac1 and Cdc42 were unaltered by the loss of RhoA, decreased activation of both Rac1 and Cdc42 was observed in cell culture (Jackson et al., 2011).

Cdc42 deficiency in keratinocytes is characterized by mouse growth retardation, impaired hair formation, hyperproliferation of interfollicular keratinocytes, defects in cell junctions and deposition of basement membrane components (Wu et al., 2006a,b).

The lack of Cdc42 altered hair follicle epithelial progenitor cells differentiation resulting in their formation into interfollicular epidermal keratinocytes (Wu et al., 2006b). This was linked to reduced nuclear beta-catenin and subsequent transcription of hair follicle specific genes. It was shown that Cdc42 is necessary for the stabilization of beta-catenin by preventing its phosphorylation through GSK3beta. This involves Par6-Par3-PKCzeta mediated phosphorylation of GSK3beta (Wu et al., 2006b). The same pathway was necessary for maintenance of mature cell-cell junctions (Wu et al., 2006b). Interestingly, it has also been shown in primary keratinocytes isolated from these mice, that Cdc42 was also necessary for the formation of mature cell junctions. This is again mediated by interaction with Par6-Par3-PKCzeta, however, independent of GSK3beta and beta-catenin (Du et al., 2009). Reduced PKCzeta phosphorylation in the absence of Cdc42 was also linked to defective basement membrane deposition (Wu et al., 2006a).

Whereas for keratinocytes, mice deficient for all three major Rho GTPases have been generated, to date, for dermal fibroblasts, only mice deficient for Rac1 have been published (Liu et al., 2008, 2009).

Rac1 deficiency in fibroblasts is characterized primarily by defects in alpha-Smooth Muscle Actin (alpha-SMA) expression, in matrix contraction and collagen production (Liu et al., 2008), as well as delayed wound closure (Liu et al., 2009). This indicates involvement of Rac1 in fibroblast activation during fibrogenesis and wound healing *in vivo*. The fact that reduction in ROS was found in the presence of Rac-1 deletion and that it was possible to partially reverse the phenotype by the addition of hydrogen peroxide, suggested that the action of Rac1 on dermal fibroblasts may be mediated by ROS production (Liu et al., 2008). In this context, it is interesting to note that ROS are one of the key factors involved in the pathogenesis of scleroderma (Yamamoto, 2009), and, that mice lacking Rac1 in fibroblasts are resistant to this condition (Liu et al., 2009).

Rho GTPases can influence production of ROS

ROS are oxygen derived chemical species that have a single unpaired electron in the outer orbit and are therefore highly reactive. ROS are produced in all aerobic organisms as a natural by-product of oxygen metabolism (e.g. the mitochondrial respiratory chain) or following some enzymatic reactions. These latter include xanthine oxidase, cyclo-oxygenases (COX) and lipoxygenases (LOX), NO synthases, and mitochondrial oxidases (Lambeth, 2004). The most common ROS produced are oxygen radicals, hydrogen peroxide,

peroxynitrite and superoxides. Given the high reactivity of ROS, maintenance of the redox balance is extremely important and this task is carried out by an antioxidant system which includes enzymes such as superoxide dismutases, peroxidases and catalases (Hordijk, 2006). However, under stressful conditions, excessive ROS production can overcome the antioxidant system resulting in oxidative stress. This is characterized by presence of large amounts of ROS that can bind non-specifically, and consequently can cause irreversible damage to cellular macromolecules including nucleic acids, sugars, lipids and proteins (Lambeth, 2004; Rada and Leto, 2008). Oxidative stress has been linked to the pathogenesis of numerous diseases (Spector, 2000) including asthma (Dozor, 2010), atherosclerosis (Vogiatzi et al., 2009), cardiovascular disease (Lakshmi et al., 2009) and cancer (Liou and Storz, 2010), inflammatory skin diseases, psoriasis and vitiligo (Sander et al., 2004; Bickers and Athar, 2006).

As stated previously, Rho GTPases have been shown to be regulators/inducers of ROS production in a number of systems, and, there is also clear indication that this could be the case in skin *in vivo* according to evidence from two wound healing studies (Sen et al., 2002; Liu et al., 2009). While Liu et al. (2009) showed that ROS production is under the control of Rac proteins in fibroblasts, this study does not establish the connection with Nox (Liu et al., 2009). Likewise the study of Sen et al. (2002), in which Rac1 overexpression in closing wounds established a link between Rac1 and Nox mediated production of ROS in skin, does not determine the cellular source of ROS (Sen et al., 2002). However, taken together, these studies provide a general indication that Nox mediated ROS production can occur in the skin *in vivo*. This connection will be discussed in more detail within the next sections.

It is reasonable to suggest that active Rho GTPase proteins are permissive in the generation of ROS as by-products. In fact Rac proteins, for example, by activating PLA_2 can facilitate release of arachidonic acid (Peppelenbosch et al., 1995; Kim and Kim 1997), which can be in turn further metabolized with the resultant generation of ROS as by-products (Tang et al., 2007; Yun et al., 2010). Interestingly, secreted metabolites of arachidonic acid can also affect surrounding cells and potentially stimulate activity of their Nox enzymes (Cho et al., 2011), whose specific activity is to produce ROS (Lambeth, 2004).

Inhibition of Rho proteins has also been reported to prevent TGFbeta induced hydrogen peroxide production *in vitro* (Koo et al., 1999). Similarly Cdc42 inhibition prevents ethanol induced production of hydrogen peroxide (Qian et al., 2003). However, the involvement of Rho GTPases in generation of ROS as by-products is beyond the focus of the present review. Our primary emphasis is on the involvement of Rho GTPases in influencing a regulated production of ROS, and, on the potential role that this can play *in vivo* in maintaining skin homeostasis.

Interestingly, Rho GTPases, which have been indicated as central mediators for regulated ROS production (see next sections), are also among the effectors of ROS (Heo, 2011). Indeed, ROS, have been shown to activate Rho/ROCK signaling pathways in a number of systems (Jin et al., 2004; Jernigan et al., 2008; Broughton et al., 2010; Chi et al., 2010), and it has been also shown that activation of RhoA can be dependent on Rac1 (Wojciak-Stothard et al., 2005). A proposed mechanism by which ROS regulate Rho GTPase activity, could be via direct oxidative action on a cysteine containing redox active motif located in the GDP binding site, whose oxidation determines release of GDP, as demonstrated in a cell free system (Heo and Campbell, 2005; Aghajanian et al., 2009). Interestingly, using the same cell free system, it was shown that for RhoA, due to the presence of an additional cysteine in this motif, a protracted oxidation may lead to a hindrance to bind GTP after the release of GDP. This would therefore potentially lead to inhibition of the pathway (Heo and Campbell, 2005). However, in cell cultures, ROS activity has been shown to stimulate both RhoA and Rac1 pathways (Aghajanian et al., 2009).

Also, Rac1 dependant ROS production has been correlated to activation of p190Rho-GAP protein, which ultimately results in deactivating Rho mediated pathways (Nimnual et al., 2003; Buricchi et al., 2007). Indeed this mechanism of RhoA deactivation has been shown to be modulated by Nox1 dependant superoxide production (Sadok et al., 2009).

Nox complexes and their regulation through Rho GTPases

Many cell types have dedicated enzyme systems which provide a regulated mechanism for intracellular generation of ROS. These enzymatic systems are known as the NADPH oxidase (Nox) complexes (Fig. 2), whose central component is a Nox protein (Lambeth, 2004; Geiszt, 2006).

Nox proteins compose a family of seven members consisting of Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Table 2). All members form a multi-subunit complex which functions in the production of superoxide, which can be rapidly dismutated into

Table 2. NADPH oxidase complex enzymes and regulatory subunits.

Enzyme	Regulatory subunits
Nox1	p22*phox*, Noxa1, Noxo1, Rac1
Nox2	p22*phox*, p40*phox*, p47*phox*, p67*phox*, Rac1/Rac2
Nox3	p22*phox*, (Regulators of Nox1 and Nox2)*
Nox4	p22*phox*
Nox5	Calcium
Duox1	Calcium
Duox2	Calcium

*activity not reliant on but increased in presence of Nox1- and Nox2-regulators

hydrogen peroxide (Lambeth, 2004; Bedard and Krause, 2007). This is achieved by the transfer of electrons from cytoplasmic NADPH across plasma and phagosomal membranes to donate them to oxygen thus forming superoxide (DeLeo et al., 1999; Lambeth, 2004; Bedard and Krause, 2007).

Rac1 and Rac2 have been found to play a central regulatory role in Nox activity and thus to substantially contribute to a regulated production of ROS primarily associating with Nox1 and Nox2 (Bokoch and Diebold, 2002; Hordijk, 2006). This association was first established by studies in phagocytic cells (Abo et al., 1991; Knaus et al., 1991). However, it was later shown to exist also in non-phagocytic cells, first identified in fibroblasts (Sundaresan et al., 1996), but most evidence comes from studies using endothelial cells (Werner, 2004; Ray and Sham, 2005; Hordijk, 2006). This model will be briefly presented here.

Nox2 (originally named gp91*phox*) was the first member described after it was identified in phagocytic cells and serves as the prototype for the remaining members of the family (DeLeo et al., 1999; Lambeth, 2004; Bedard and Krause, 2007). The active Nox2 complex consists of a catalytic flavo-heme subunit Nox2, and regulatory subunits; p22*phox*, p67*phox*, p47*phox* and p40*phox* as well as a member of the Rac subfamily, either Rac1 or Rac2 (DeLeo et al., 1999; Lambeth, 2004; Bedard and Krause, 2007). In cell free systems, i.e., macrophage membranes (Abo et al., 1991) and neutrophil cytosolic fraction (Knaus et al., 1991), it has been shown that either of these Rac isoforms can activate this Nox complex (Abo et al., 1991; Knaus et al., 1991). However, phagocytic cells preferentially act through Rac2 (Dorseuil et al., 1992; Heyworth et al., 1994; Voncken et al., 1995; Roberts et al., 1999). Indeed deletion of Rac2 reduces Nox2 activity in neutrophils (Roberts et al., 1999; Kim and Dinauer, 2006). In resting neutrophilic cells, Nox2 and p22*phox* form the membrane bound heterodimer flavocytochrome b_{558}. Nox2, i.e., the beta-subunit of this heterodimer, is a 65kDa protein, but runs in SDS-PAGE gels at 91kDa due to its glycosylation (Groemping and Rittinger, 2005). Nox2 consists of six transmembrane alpha-helices, a FAD binding domain, a NADPH binding domain, and four heme-binding histidines, and therefore contains all the necessary components for electron transfer from NADPH to oxygen (Groemping and Rittinger, 2005). The α-subunit of flavocytochrome b_{558}, i.e. p22*phox*, is a 21kDa protein consisting of three transmembrane alpha-helices and contains a proline rich region that can bind the two SH3 domains of p47*phox* (Groemping and Rittinger, 2005). The remaining subunits reside in the cytosol, i.e. Rac2 in its inactive form, as well as p67*phox*, p47*phox*, and p40*phox*, which form a cytosolic regulatory complex (DeLeo et al., 1999; Lambeth, 2004; Bedard and Krause, 2007).

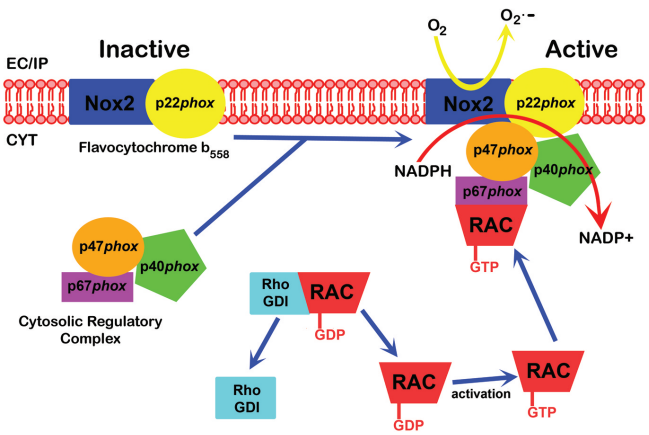

Fig. 2. Model of Activation of NADPH Oxidase Complex in Neutrophils. In resting Neutrophils, the Nox2 (formerly known as gp91*phox*, dark blue) and p22*phox* (yellow) subunits form the membrane bound flavocytochrome b_{558}. The p47*phox* (orange), p40*phox* (green) and p67*phox* (purple) form a cytosolic regulatory complex. Rac proteins (red) reside in the cytosol in an inactive GDP-bound state, and sequestred by Rho-GDI proteins (light blue). Upon activation, the cytosolic regulatory complex translocates to the membrane and binds to the flavocytochrome b_{558}. Rac proteins translocate separately following conversion into their active GTP-bound state (see Fig. 1) and bind to the p67*phox* subunit. This completes the formation of the Nox2 complex, which has now been activated. The Nox2 subunit contains all the necessary components for the binding and transfer of electrons from NADPH to O_2 resulting in the formation of superoxide (O_2·-). Drawn modified according to Lambeth (2004).

The Nox complex is activated in phagocytic cells upon internalization of a pathogen or in response to inflammatory signals. Following this, the cytosolic regulatory complex (i.e. p67*phox*, p47*phox*, and p40*phox*) translocates to the membrane and binds to the flavocytochrome b_{558}. At the same time Rac2 is converted to its active form and completes the assembly of the Nox complex. Electrons can now be transferred through the complex to produce superoxide for the destruction of pathogens (DeLeo et al., 1999; Lambeth, 2004; Bedard and Krause, 2007).

As discussed previously, Nox mediated ROS production is not limited to phagocytic cells. Non-phagocytic cells, including neurons, hepatocytes, myocytes and endothelial cells, can also produce ROS via Nox complexes, although to a much lesser extent (Bedard and Krause, 2007). ROS production via Nox complexes has been implicated in redox dependant regulation of signaling pathways of cell growth, differentiation, migration, angiogenesis as well as inflammation, vascular hypertrophy and atherosclerosis (Bedard and Krause, 2007). Production of ROS during these processes can occur in response to extracellular stimuli such as binding of growth factors, cytokines, and extracellular matrix molecules to their membrane receptors (Hordijk, 2006).

A number of Nox2 homologues have been identified. They now form the Nox family, all of which share structural features (such as six transmembrane alpha-helices, a FAD binding domain, a NADPH binding domain, and four heme-binding histidines), but they differ in their regulation, tissue distribution and

expression (Groemping and Rittinger, 2005; Quinn et al., 2006).

Nox1 has 56% sequence identity to Nox2 and is similar in size (55-60kDa) to the de-glycosylated form of Nox2 (Groemping and Rittinger, 2005; Bedard and Krause, 2007). The Nox1 gene encodes an alternative splice variant which lacks exon 11 and does not encode a functional oxidase (Banfi et al., 2000; Geiszt et al., 2004).

A high level of expression of Nox1 is found in the epithelial layer of the colon where it is thought to function in host defense (Suh et al., 1999; Geiszt et al., 2003a). Low levels of expression are also found in the prostate, uterus and vascular smooth muscle (Suh et al., 1999). The p22*phox* subunit, an essential component of the membrane bound flavocytochrome b_{558} found in phagocytic cells, has also been found to be expressed in a number of non-phagocytic cells including skeletal muscle, fibroblasts, and endothelial cells (Quinn et al., 2006; Bedard and Krause, 2007). Additionally, p22*phox* has been proven to be essential for the Nox generation of ROS also for Nox1, as well as Nox3 and Nox4 (Parkos et al., 1988; Suh et al., 1999). Cytosolic regulators of Nox1 in non-phagocytic cells were first identified in colon tissue (Banfi et al., 2003; Geiszt et al., 2003b; Takeya et al., 2003). These regulators are termed Noxa1 (Nox activator 1) and Noxo1 (Nox organizer 1), which are the homologues for the p67*phox* and p47*phox* subunits respectively that are found in phagocytic cells (Banfi et al., 2003; Geiszt et al., 2003b; Takeya et al., 2003).

Rac1 has been shown to bind to Noxa1. Binding of Rac1 is restricted to its GTP-bound status, whereas GDP-bound Rac1 does not bind to Noxa1. Therefore, it is likely that active Rac1 may be involved in the formation and activation of the Nox complex in non-phagocytic cells (Takeya et al., 2003).

Nox3 expression is limited to the inner ear where it plays a function in balance and gravity perception (Paffenholz et al., 2004). It bears close resemblance to both Nox1 and Nox2. It is similar in size being approximately 55-60kDa and has 58% sequence homology to Nox2 (Groemping and Rittinger, 2005; Bedard and Krause, 2007). However, unlike Nox1 and Nox2, Nox3 can remain active in the absence of any activators or co-factors (including Rac proteins), but its activity is increased in the presence of the regulators of Nox1 or Nox2 (Geiszt et al., 2000).

Nox4 has 39% sequence homology to Nox2, and shows highest expression in the kidney, where it is thought to function as an oxygen sensor (Shiose et al., 2001). Antibodies for Nox4 recognize two bands on SDS-PAGE gels, one at 65kDa and the other between 75-80kDa that is not due to glycosylation (Shiose et al., 2001; Hilenski et al., 2004; Hwang et al., 2005). Four splice variants of Nox4 have been reported (Goyal et al., 2005) and this may account for the two different sizes of the protein. Whereas Nox4 is not regulated by the cytosolic components of Nox1 and Nox2, it does require p22*phox* for stabilization (Geiszt et al., 2000; Rada and Leto, 2008). There is some evidence to suggest that Rac proteins may affect its activation indirectly, but the exact mechanism is not established (Gorin et al., 2003; Wu et al., 2007; Chai et al., 2008).

Nox5 shows only 27% sequence homology to Nox2 and its expression is restricted to the spleen, lymph nodes and testis (Banfi et al., 2001; Bedard and Krause, 2007). In contrast to Nox2, it contains an intracellular EF-hand domain for Ca^{2+} binding, resulting in a protein of 85kDa (Banfi et al., 2000, 2001; Brar et al., 2003).

Duox1 and Duox2 were first identified in the thyroid during a search for the source of hydrogen peroxide that is required for thyroid hormone synthesis (De Deken et al., 2000), and their name derives from the fact that they are dual oxidases. In addition to their Nox homology domain, which has 50% sequence homology to Nox2, they contain a transmembrane peroxidase homology domain in the NH_2 terminus (De Deken et al., 2000; Bedard and Krause, 2007). Between these two domains they contain two EF hand domains giving them a total size of 160kDa in the non-glycosylated form (De Deken et al., 2002). Duox1 and Duox2 are also expressed in lung, colon and rectal epithelia layers and in exocrine glands where they may function in the production of hydrogen peroxide for host defense (Geiszt et al., 2003c).

There is little published information on the regulation of Nox5, Duox1 and Duox2, except that their activity is affected by intracellular calcium levels (Leto et al., 2009). However, it is known that they are not dependent on Rac proteins or on the other regulators described for Nox2 and also depicted in Fig.2 (Fortemaison et al., 2005; Geiszt, 2006; Montezano et al., 2010).

Although Rac proteins are the main Rho GTPases involved in controlling activity of Nox1 and Nox2, a role for RhoA in modulating Nox activity has been suggested as well. While not directly controlling Nox activity in cell free systems, inhibition of RhoA in cell cultures hindered phosphorylation of p47*phox* leading to a decrease in superoxide production (Kim et al., 2004, 2005).

In summary, Nox1 and Nox2, are therefore the only members of the Nox family, for which the participation of Rho GTPases as a part of the complex has been clearly established. Although some more peripheral regulatory involvement of RhoA has been indicated, the Rho GTPases which become integrated in the Nox complexes and are central in their activation are Rac proteins. The original prototype for these interactions is represented by the model shown for the Nox2 complex in neutrophils (Fig. 2), whereas for epithelial and/or fibroblastic cells a model containing Nox1 and homologous regulatory factors has been proposed.

Whether this latter model can be translated to skin tissue *in vivo*, is yet to be fully elucidated. However, there are data indicating that skin cells are capable of a regulated ROS production via Nox1, and, there is also

evidence to support a link to Rho GTPases in this process.

Nox and Skin

HaCaT cells have been used extensively to study Nox complexes in keratinocytes (Goldman et al., 1998, 1999; Sen et al., 2002; Chamulitrat et al., 2004; Grange et al., 2009). Increase in ROS production in HaCaT cells, following stimuli such as EGF, bradykinin, Ca^{++}-ionophore A23187, Platelet activating factor, and lysophosphatidic acid, can be prevented by inhibiting Nox complexes (Goldman et al., 1998, 1999), indicating a Nox mediated ROS production in this keratinocyte cell line *in vitro*. Indeed evidence has been provided that these cells have been shown to contain Nox1, p40*phox*, p67*phox*, and p22*phox*, and, also express mRNA for Nox1, Nox2 and Nox4 (Chamulitrat et al., 2004). In cell free assays, membranes positive for p22*phox* are capable of producing a low level of superoxide in a constitutive manner (Chamulitrat et al., 2004). HaCaT cells also contain Rac1 (Chamulitrat et al., 2004), which in other epithelial cells has been shown to be a component of the Nox1 complex (Miyano et al., 2006; Nisimoto et al., 2008; Comstock et al., 2011). Interestingly, in HaCaT cells, the introduction of active Rac1 has been shown to increase VEGF protein expression in a Nox-dependent manner (Sen et al., 2002). This points at a regulatory role for Rac1 in the control of a cellular pathway of primary importance via Nox mediated ROS production, at least in this *in vitro* model.

More recently, it has been shown that treatment of HaCaT cells with *Propionibacterium acnes* resulted in the production of large amounts of ROS which were prevented by silencing Nox1 (Grange et al., 2009). All these data fundamentally suggest that HaCaT cells possess an actively functioning Nox1 complex (but do not exclude the presence of further Nox complexes). It is, therefore, likely that Nox activity plays a major role in regulating ROS production in these cells, both at basal level and in response to various stimulators.

Evidence for a functioning Nox1 complex has also been provided in primary human keratinocytes (Wu et al., 2008). These cells express Nox1, p22*phox*, and the cytosolic components; p47*phox*, p67*phox*, as well as Noxo1 (Wu et al., 2008). Additionally, following UVA irradiation, these keratinocytes, showed an increase in phosphorylation of p47*phox* (Wu et al., 2008), which is indicative of Nox activity (Lambeth, 2004; Geiszt, 2006; Bedard and Krause, 2007). This increase in ROS production was sensitive to inhibition by DPI (Wu et al., 2008), thus indicating Nox mediated ROS production in response to UVA.

There are a considerable number of studies on dermal fibroblast in which production of ROS in response to different stimuli has been analyzed. These include UV irradiation (Brenneisen et al., 2002), PLA2 (Kim et al., 2009), TGFbeta (Choi et al., 2009), and TNFalpha (Kohler et al., 1999). Additionally, dermal fibroblasts have been extensively used as a model to investigate the effects of antioxidants for the reduction of ROS in the skin (Ramachandran et al., 2010; Kim et al., 2011; Pallela et al., 2011). Some studies also presented evidence indicating Nox activity as a source of ROS production in dermal fibroblasts (Dhaunsi et al., 2005; Svegliati et al., 2005; Choi et al., 2009; Dooley et al., 2010). Expression of Nox4 in dermal fibroblasts has been reported both at the mRNA (Jun and Lau, 2010; Loughlin and Artlett, 2010) and at the protein level (Chenevier-Gobeaux et al., 2006). Interestingly, expression of Nox1 and of Nox2 appears to be dependent on culture conditions (Dhaunsi et al., 2005; Chenevier-Gobeaux et al., 2006; Jun and Lau, 2010; Loughlin and Artlett, 2010). In has also been shown that, increase in Nox1 activity in dermal fibroblasts triggered by CCN1 *in vitro*, correlates to activation of Rac1 (Jun and Lau, 2010).

Generation of ROS via Nox complexes is not limited to keratinocytes and dermal fibroblasts. It has in fact been described that in proliferating melanocytes, along with mRNA expression for Nox4, there is expression for p22*phox*, but, not for Nox1, Nox2, p47*phox* or p67*phox* (Brar et al., 2002). In contrast, no specific description of Nox complexes in Langerhans cells has been reported. This is surprising as Langerhans cells are dendritic cells that play important roles in host defense (Kissenpfennig et al., 2005), and, in this cell type in general, Nox2 derived ROS have been reported to play a role in numerous normal cellular functions (Grandvaux et al., 2007),

Whereas the *in vitro* studies discussed in this section provide an indication for potential cellular sources of Nox activity in skin, the studies of Sen et al. (2002) and Roy et al. (2006) suggest the presence of a functional Nox complex *in vivo*. Sen et al. (2002) showed that effects due to overexpression of active Rac1 were reverted by DPI, indicating mediation through Nox activity (Sen et al., 2002). In this way, this study, not only indicates presence of a functional Nox complex in skin *in vivo*, but it also points at a connection with Rac1, at least when this Rho GTPase is artificially overexpressed in skin. Roy et al. (2006) showed that p47*phox* KO mice have impaired wound healing due to an inability to produce ROS which was mitigated by hydrogen peroxide (Roy et al., 2006). The dysfunctional p47phox, combined with reduced ROS production, suggest a missing activity of the Nox complex in these KO mice. Despite the fact that the cellular sources are not specifically analyzed, these papers point out at the fact that functional regulation of Nox complexes is important in skin homeostasis, and, to some extent also anticipate the link with the role of Rac1 in this context.

How much can ROS production in skin be connected with Rho GTPases *in vivo*?

This section will now explore the possibility that some of the effects of Rho GTPase deletion in skin could potentially be contributed by defective ROS production, which would otherwise normally occur *in vivo*.

ROS, Rac, ERK and keratinocytes

Keratinocytes under hyperproliferative conditions, and in the absence of Rac1, show defects in the ERK signaling pathway (Tscharntke et al., 2007; Wang et al., 2010).

In HaCaT cells, it has been shown that UVB exposure, as well as increasing ROS production, also stimulates ERK phosphorylation, and this in turn is necessary for MMP-1 expression (Kim et al., 2010). The authors reported that UVB induced MMP-1 expression was abolished if ERK phosphorylation or the Nox complex were inhibited (Kim et al., 2010). An earlier study by Gupta et al. (1999) provides an additional link between ERK and ROS using a papilloma keratinocyte cell line undergoing malignant transformation. These malignant cells had increased ROS levels. This contributed to an increase in cell proliferation. These cells also had increased ERK-1/2 (and p38 MAPK) phosphorylation, which paralleled the progression of the malignancy and was reduced by treatment with the ROS scavenger NAC (Gupta et al., 1999). Both these studies indicate the need of ROS for ERK phosphorylation in keratinocytes.

However, ERK phosphorylation seems to be a prerogative of hyperproliferative conditions, since no ERK alterations were found in non-hyperproliferative keratinocytes, even in the absence of Rac1 (Chrostek et al., 2006). Also, the above studies (Tscharnke et al., 2007; Gupta et al. 1999; Wang et al., 2010,; Kim et al., 2010) independently link the defect in ERK phosphorylation with absence of Rac1 and also with defective Nox activity, but they do not provide a direct link between the three (ERK, Nox and Rac1) in the skin. Rygiel et al. (2008) provide a more direct piece of evidence for a possible link between Rac proteins, ROS and ERK phosphorylation, where the three factors could be part of a sequential cascade of events. *In vitro* inactivation of Tiam1 in keratinocytes correlated with reduction in Rac activity and increased the susceptibility to apoptosis due to defective ERK phosphorylation and failure of the corresponding cell survival pathway (Rygiel et al., 2008). Interestingly the authors also described defective ROS production within the cells (Rygiel et al., 2008).

By introducing exogenous expression of catalytically active Tiam1 to the Tiam1-deficient keratinocytes, the ROS levels after stress were restored to that of the control cells, and survival was improved. Conversely, using a mutant Tiam1 (unable to activate Rac), such rescue did not occur. This shows dependency of ROS production on Rac activity in this system. At the same time, the use of Nox complex inhibitors and antioxidants in control keratinocytes caused a simultaneous reduction in the production of ROS and ERK phosphorylation, which indicates a direct link between the two. Therefore, at least under these conditions, a sequential link following the order: Tiam1, Rac activation, Nox mediated ROS production, ERK phosphorylation, and eventually cell survival, can be recognized (Rygiel et al., 2008). This could suggest that ERK phosphorylation *in vivo*, when keratinocytes are undergoing a stressful situation (e.g. wound or tumorigenic stimulation) may follow a similar pathway, and, eventually influence cell survival.

ROS, Rho and phosphorylation of myosin light chain

RhoA inhibition in keratinocytes results in the decreased phosphorylation of myosin light chain (Jackson et al., 2011). RhoA, although not being a direct activator, can also be involved in controlling Nox mediated ROS production through phosphorylation of the cytosolic regulatory factor p47*phox* (Kim et al., 2004, 2005).

ROS have been extensively shown to increase the phosphorylation of myosin light chain in endothelial cells (Lopez-Ongil et al., 1999; Haorah et al., 2005; Kuhlmann et al., 2007) and airway smooth muscle (Samb et al., 2002), and there is evidence that at least in part, the source of ROS is a Nox complex (Kuhlmann et al., 2007). Along this line, it could also be hypothesized that this may be the case in keratinocytes, although no report of such an investigation exists.

ROS, Fibroblast differentiation and collagen synthesis

It was shown that lack of Rac1 in fibroblasts results in impairment in fibroblast activation, collagen production, and wound healing (Liu et al., 2008, 2009). The authors also reported that this was mitigated by addition of hydrogen peroxide (Liu et al., 2009). This strongly indicates that in dermal fibroblasts, for normal wound healing, Rac1 exerts its effects through a ROS dependent mechanism (Liu et al., 2008, 2009). In line with this, Xu et al. (2009) reported that inhibition of Rac1 in dermal fibroblasts isolated from scleroderma patients, reversed the scleroderma phenotype in these cells (Xu et al., 2009). It is well established that ROS are one of the key factors causing scleroderma (Parke and Sapota, 1996; Yamamoto, 2009). It has been reported that ROS production in fibroblasts from scleroderma patients is derived from Nox complex activation (Sambo et al., 2001). Moreover, the general involvement of Nox complexes in myofibroblast differentiation and subsequent extracellular matrix production has been extensively reviewed recently (Barnes and Gorin, 2011). These considerations on Nox involvement in fibroblast activation would suggest that reduction in ROS production in dermal fibroblasts during wound healing, as reported in absence of Rac1 (Liu et al., 2009), might at least in part be due to a decrease in Nox activity.

Altogether, these studies of Sambo et al. (2001), Xu et al. (2009), and Liu et al. (2009) provide a further link which underpins the concept of the involvement of Rho GTPases in a regulated cutaneous production of ROS.

Concluding remarks

The involvement of Rac proteins in the production of ROS is well established *in vitro*. Within the last ten years evidence has come to light that also other members of the Rho GTPase family, namely Rho proteins and Cdc42, can play a role in ROS production. Interestingly, whereas members of the Rho GTPase family affect ROS production, ROS can in turn affect the activation state of members of the Rho GTPase family.

To date, no *in vivo* study exists that directly connects Rho GTPases with Nox mediated production of ROS in skin. The challenge for the future will be to investigate the *in vivo* importance of the Rho GTPase-ROS relationship during skin development, skin maintenance, and particularly in skin diseases.

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