Mini Review

Study designs to investigate Nox1 acceleration of neoplastic progression in immortalized human epithelial cells by selection of differentiation resistant cells☆

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A B S T R A C T
To investigate the role of NADPH oxidase homolog Nox1 at an early step of cell transformation, we utilized human gingival mucosal keratinocytes immortalized by E6/E7 of human papillomavirus (HPV) type 16 (GM16) to generate progenitor cell lines either by chronic ethanol exposure or overexpression with Nox1. Among several cobblestone epithelial cell lines obtained, two distinctive spindle cell lines – FiB and NuB1 cells were more progressively transformed exhibiting tubulogenesis and anchorage-independent growth associated with increased invasiveness. These spindle cells acquired molecular markers of epithelial mesenchymal transition (EMT) including mesenchymal vimentin and simple cytokeratins (CK) 8 and 18 as well as myogenic alpha-smooth muscle actin and caldesmon. By overexpression and knockdown experiments, we showed that Nox1 on a post-translational level regulated the stability of CK18 in an ROS-, phosphorylation- and PKCε-dependent manner. PKCε may thus be used as a therapeutic target for EMT inhibition. Taken together, Nox1 accelerates neoplastic progression by regulating structural intermediate filaments leading to EMT of immortalized human gingival epithelial cells.

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Contents

Introduction .................................................................................................................................................. 140
Role of ROS and NADPH oxidases in epithelial cancers ............................................................................. 141
Setting up model human epithelial cells to study Nox1 functions ................................................................. 141
Selection of preneoplastic human epithelial cells by differentiation resistance ......................................... 141
Ethanol increases neoplastic progression of GM16 to cells expressing Nox1 ............................................. 141
Nox1 overexpression renders differentiation resistance for selection of preneoplastic cells ................... 142
Limitations and pitfalls of a method of preneoplastic selection .................................................................. 143
Gain of survival markers in selected preneoplastic cells .......................................................................... 143
Epithelial mesenchymal transition (EMT) of Nox1–preneoplastic cells ...................................................... 144
Regulation of intermediate filaments by Nox1 ............................................................................................... 144
Role of PKCε in CK18 accumulation by Nox1 ............................................................................................ 144
Role of Nox1 in accelerating transformation beyond immortalization steps ............................................. 145
Acknowledgments ....................................................................................................................................... 146
References .................................................................................................................................................. 146

Abbreviations: AIG, anchorage-independent growth; CK, cytokeratin; EGF, epidermal growth factor; EMT, epithelial mesenchymal transition; GM, gingival mucosal; HPV, human papillomavirus; IAP, inhibitor of apoptosis protein; iNOS, inducible nitric oxide synthase; MEF2, myocyte enhancing factor 2; MMP, matrix metalloproteinases; Nox, NAD(P)H oxidase; PMA, 12-O-tetradecanoylphorbol-13-acetate; ROS, reactive oxygen species
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Introduction

This mini-review illustrates our research approach to investigate the role of NADPH oxidase homolog 1 (Nox1) in neoplastic progression of human epithelial cells. It is known that a direct tumorigenic conversion of human epithelial cells to cancer phenotype is very difficult, and impossible to achieve in tissue cultures. Our experimental designs described below have provided new insights that Nox1 plays a role in regulation of epithelial mesenchymal transition (EMT) on the molecular and functional levels.

Role of ROS and NADPH oxidases in epithelial cancers

The implication for reactive oxygen species (ROS) to play a role in epithelial cancers has long been recognized based on the fact that human cancer cells produce high levels of $H_2O_2$ [1], and superoxide radical mediates neoplastic transformation of mouse keratinocytes [2]. Data in the last 14 years have shown that the sources of ROS in non-phagocytic epithelial cells are the homologs of gp91phox, the catalytic subunit of the respiratory burst oxidase of phagocytes, so-called Nox consisting of Nox1, Nox3, Nox4 and Nox5 (while gp91phox is Nox2) [3,4]. These Nox proteins are ~63–65 kDa in size and show 20–60% sequence identity with one another. The first sequence of Nox cDNA from 3 laboratories was all cloned from normal human colon initially called Mox1 (mitogenic oxidase1) and later renamed as Nox1 [5–7].

Nox1, 2, 4, and 5 mRNAs are expressed in a variety of human cancer cell lines indicating an association with cancer development [3]. The role of Nox1 in cancer was first shown by growth stimulation and tumorigenic conversion of rodent NIH3T3 fibroblasts upon Nox1 overexpression [5]. This tumorigenic phenotype was reversed by overexpression of catalase indicating that $H_2O_2$ generated secondarily Nox1 serves as transformation signals [8]. Nox1 acting as a mitogenic oxidase was further confirmed by data showing Nox1 as a regulator of cyclin D1 [9], and that Nox1 produces $H_2O_2$ via Rac1 upon epidermal-growth-factor (EGF) receptor ligation [10].

The role of Nox1 in human epithelial cancers was first reported by showing that overexpression of Nox1 increased tumorigenic potentials of DU-145 human prostate cancer cell line [11]. While Nox1 mRNA is present in both primary and transformed human colon epithelial cells [12], Nox1 mRNA is detected only in human colon tumors but not in ovarian, lymph, breast, prostate, lung, brain, and melanoma tumors [13]. Nox1 is strongly expressed in gastric adenocarcinomas, colonic adenomas and well differentiated adenocarcinomas [14], and breast and ovarian tumors [15]. However, a study in human colon reported that Nox1 was constitutively expressed in colonic epithelium but was not associated with colonic tumorigenesis [16]. These data did not support the role of Nox1 in cancer but rather in inflammation as Nox1 protein is also present in lymphocytes in inflammatory bowel disease.

Setting up model human epithelial cells to study Nox1 functions

Transformation and tumorigenic conversion of mouse cells, such as, keratinocytes [2] and fibroblasts [5] could be accomplished in one step by overexpression of an oncogene or oncogene-like such as Nox1. Human epithelial cells are highly resistant to transformation even after treatment with carcinogens [17,18]. Tumorigenic conversion and transformation of human epithelial cells can be achieved in a multi-step fashion, whereby immortalization is the critical step to obtain tumorigenicity [19,20]. This accounts for the difficulties to perform molecular studies on tumorigenic conversion of human cells, and in fact many studies utilize carcinoma cell lines. In the latter case, the mechanisms for an early stage of cell transformation cannot be studied. Therefore, we had taken a step in choosing a non-cancer immortalized cell line to study the role of Nox1 on phenotypic changes associated with preneoplastic progression beyond immortalization.

It is known that HPV16E6 is able to immortalize human keratinocytes [21], and confers resistance against radiation stress [22]. In our laboratory, we were able to successfully immortalize human gingival mucosal (GM) keratinocytes with human papillomavirus (HPV) type 16 E6/E7 oncogenes to generate a so-called GM16 cell line [23]. Immortalization by HPV has been shown to be prerequisite for progression of cell transformation elicited by subsequent exposure to a carcinogen, such as, benzo(a)pyrene [24], asbestos [25], cisplatin [26], and cigarette smoke condensate [27].

About the same time as the discovery of Nox1 [5], an earlier biochemical characterization of ROS generation by human epithelial cells was already described in human skin keratinocyte HaCaT cells during growth-factor activation [28] and overexpression of Ras [29]. In 2004, we accordingly have reported that HaCaT cells express Nox1, Nox2, and Nox4 at mRNA levels, and that Nox1 appears to be the major gp91phox homolog expressed on the protein level [30]. HaCaT cells carry genetic abnormalities from spontaneous immortalization and were identified as pre-transformed. HaCaT as an immortalized cell line proliferate in standard DMEM. We showed that HaCaT cells expressed Nox1 protein more than GM16 keratinocytes which proliferate only in low-calcium keratinocyte growth medium (KGM) [30].

Selection of preneoplastic human epithelial cells by differentiation resistance

Defective terminal differentiation in cell cultures is regarded as a consistent and selectable character of neoplastic human keratinocytes [31], and considered as an initiation of carcinogenesis [32]. In experiments using cultured cells, resistance against calcium-induced terminal differentiation has been used for selection of preneoplastic cells [33,34]. Indeed, preneoplastic cells induced by Ras [33], HPV16E6 [34], or treatment of 12-O-tetradecanoylphorbol-13-acetate (PMA) [35], exhibit an inhibition of terminal differentiation. Therefore, we utilize a criterion in selecting cells that are resistance against calcium-induced differentiation in our experiment.

Ethanol increases neoplastic progression of GM16 to cells expressing Nox1

As HPV16-immortalized human epithelial cells have been used to study tumorigenic potential of carcinogens [24–27], we were interested in whether ethanol could further neoplastic progression of HPV16-immortalized gingival mucosal epithelial keratinocytes. In our first study, we exposed GM16 cells to 30 mM ethanol in KGM a closed small incubator with once per week ethanol replenishment for 9 weeks [23]. Many cells were initially died off when cultured medium was changed from KGM to standard high-calcium DMEM containing 10% serum without ethanol. Few selectable cells persisted and finally proliferated again to confluent cultures. Upon further 15 passaging in DMEM, mixed population of cobblestone and elongated progenitor cells were obtained. By differential trypsinization, two distinct cell populations were obtained named as EPI and FIB cell lines with epithelium-like cobblestone and elongated morphology, respectively (Fig. 1A). These EPI and FIB cells selected to survive ethanol-dependent stress and proliferating well in DMEM were considered more transformed than the parental GM16 cells. FIB cells were fibroblast-like cells showing EMT also exhibited anchorage-independent growth (AIG), and thus considered as
more transformed than EPI cells [23,36]. FIB cells at passages < 22 were able to revert back to EPI phenotype upon adaptation back to KGM indicating that the phenotype was unstable at this stage [36]. Passaging FIB cells to passages > 43 generated stable cells being unable to revert back to cobblestone morphology. Subcutaneous injection of EPI or FIB cells (at 60–70 passages) in nude mice did not form tumors, thus they were in an intermediate benign stage, but not yet tumorigenic as only FIB cells produced benign cysts [36]. Using this cell model, we reported that the more transformed FIB cells expressed increased Nox1 protein than less transformed EPI [36]. In an NADPH-reconstituted assay, plasma membranes isolated from FIB cells produced increased superoxide radical adducts compared to those from EPI cells. Thus, Nox1 expression is associated with cell transformation, i.e., elongated spindle phenotype and AIG [36].

Furthermore, we showed that FIB cells expressed increased levels of redox-sensitive inducible nitric oxide synthase (iNOS) [37]. iNOS is shown to be associated with tumor invasiveness consistent with increased iNOS at mRNA and protein levels in FIB cells. The morphological reversion of FIB cells at low passages upon adaptation to grow in KGM was associated with the reversal of vimentin expression as well as AIG. Moreover, these revertants showed reduced levels of iNOS mRNA as well as MAP kinase ERK and phospho-ERK protein expression, while FIB cells without reversion maintained the expression. The MEK1/2 inhibitor U0126 could reduce detectable iNOS mRNA levels suggesting that MAP kinases were upstream regulators of iNOS transcription. U0126 caused both morphological and functional reversion of FIB cells indicating involvement of MAP kinases in these functions [37].

**Nox1 overexpression renders differentiation resistance for selection of preneoplastic cells**

To address a direct impact of Nox1 on cell transformation, we performed transfection of GM16 cells with Nox1 plasmid [38]. In a similar manner as chronic ethanol treatment, we exposed transfected cells to high-calcium DMEM for selection of preneoplastic cells following stationary cultures in low-calcium KGM (Fig. 1B). In Nox1-transfected cells, we observed resistant cells as small fast-dividing cells after exposure with DMEM and serum for 7–10 days. These cells grew rapidly into empty spaces as differentiated cells peeled off. After 3 weeks to allow confluent cultures, resistant cells could be passaged normally to obtain...
Nox lines. Unlike primary human keratinocytes, Nox1-expressing HaCaT, EPI, and FIB cells proliferate in standard DMEM. This made it possible for us to test whether Nox1 expression confers resistance against differentiation induced by high calcium present in DMEM and serum [31–35]. We were able to obtain five stable Nox1 transfectants NuB1, FuB1, NuB2, FuB2, and NuB3 (named according to Nucleofection or FuGene6 transfection method and Batch number) (Fig. 1B). In the first round of DMEM exposure, we obtained NuB1 cell line which exhibited spindled EMT morphology and acquired AlC. In subsequent rounds, the rest of the four Nox1 transfectants produced cells with cobblestone morphology (Fig. 1B).

**Limitations and pitfalls of a method of preneoplastic selection**

Selection of preneoplastic human cells in cultures has been successfully utilized in oncogene- or HPV16-immortalized cells [33,34] rendering them to be susceptible for neoplastic progression by carcinogens [24–27]. In our work, control experiments, i.e., no ethanol treatment or control plasmid transfection were performed in parallel. Without chronic ethanol treatment, we did not obtain any progenitor cells. However, in control plasmid transfection prolonged exposure in DMEM was necessary to generate resistant cells which exhibited very slow growth rates at passages < 20, and became more proliferative at passages > 20 [38]. These cells were regarded as unusual cells which do not undergo terminal differentiation in culture previously described in human skin keratinocytes [39]. Thus, appropriate controls have to be performed for testing overexpression of a proposed oncogene or carcinogen. The limitations for selection in DMEM include an extended length of time to remove dead cells, and that the cultures should be kept stationary to near confluence prior to trypsinization. This may take as long as 3, 6 and 10 weeks for Nox1-overexpression experiments [38].

**Gain of survival markers in selected preneoplastic cells**

We further showed that preneoplastic cells by ethanol exposure or Nox1 overexpression acquire increased cell survival markers. Cyclin kinase inhibitor p16INK4a is associated with by-pass senescence and immortalization. Cyclin kinase complexation is known to be altered by E6/E7 oncoproteins of HPV16, and thus increases of p16INK4a are commonly observed in HPV16-containing cancer tissues [21,23]. As shown in Fig. 2A, p16INK4a was upregulated in NuB1 line compared with cobblestone cell lines and FIB cells harvested at equivalent passages. Ethanol-induced resistant EPI and FIB cells showed an upregulation of p16INK4a compared with parental GM16 cells [23]. Protein expression of another cyclin kinase inhibitor p27KIP1 was increased in NuB1 cells compared with cobblestone cells [40]. Survivin is a protein in a family of inhibitor of apoptosis protein (IAP), and its increased expression is associated with early stage of transformation, and during development of malignancy. NuB1 line expressed increased survivin protein compared with other Nox1 cobblestone lines (Fig. 2A). In a similar manner, NuB1 cells expressed increased clAP-1, clAP-2 as well as anti-apoptosis family member Bcx, and Bcl-2 proteins compared with cobblestone Nox lines [40]. Our data hence provide an evidence for survival advantage of spindle fibroblast-like cells over cobblestone cells.

We analyzed expression of Nox-based adapter proteins in our cell lines [41–43]. Nox1-derived cell lines showed greater mRNA expression of Nox1, Rac1, p47phox, p67phox, NOXO1, and NOXA1 concomitant with increased superoxide generation [38]. Western analysis of p47phox and p67phox of these lines is shown in Fig. 2B. All Nox-derived cell lines lacked p47phox protein expression suggesting that the Nox-derived cell lines may utilize NOXA to replace p47phox’s function. While other cobblestone lines expressed p67phox, the spindle NuB1 and FIB cells did not express p67phox indicating that these cells may utilize NOXO1 instead of p67phox for Nox1 activation. This finding gives insights into

![Fig. 2.](image-url) Expression levels of cell cycle, cell survival and Nox1 adapter proteins among spindle and cobblestone cells. (A) NuB1 cells expressed p16INK4a (top), and survivin (bottom) at higher levels than other cobblestone cell lines and FIB cells. Right 2 panels show quantification of p16INK4a/β-actin and survivin/β-actin ratio among 3 cobblestone and 2 spindle cell lines. (B) Western blot analyses of p47phox (top) and p67phox (bottom) show the absence of p47phox in all Nox lines, while p67phox was absent only in spindle NuB1 and FIB cells. A right panel shows quantification of p67phox/β-actin ratio among 3 cobblestone and 2 spindle cell lines.
specificity towards NOX1A for preneoplastic epithelial cobblestone cells, and that towards NOXO1 for spindle elongated FIB and NuB1 cells for Nox1 activation [41–43].

Epithelial mesenchymal transition (EMT) of Nox1–preneoplastic cells

Two sets of cell lines were hence generated as model systems to study an impact of Nox1 on neoplastic progression – EPI and FIB from chronic ethanol exposure [23,36,37], and five Nox1 cell lines with spindle NuB1 cells from Nox1 overexpression [38]. The spindle FIB and NuB1 cells exhibiting EMT were compared with their control counterparts. Our more recent data demonstrate the progressive steps of FIB and NuB1 cells in cell migration, invasion, and metastatic potentials.

Nox1 overexpression causes branching tubulogenic potential previously reported for sinusoidal endothelial cells [44]. Branching morphogenesis is commonly used to characterize breast and prostate cancer cells. Upon resistance against Matrigel-induced differentiation, FIB and NuB1 cells formed branching tubulogenesis with cords lacking lumen, while corresponding cobblestone cells did not [44,45]. Using invasion/migration Boyden chamber, FIB and NuB1 cells were more invasive with increased migratory capacity than control cobblestone cell lines [45,46].

EMT of FIB and NuB1 cells was associated with the loss of epithelial marker E-cadherin as well as the gain of mesenchymal marker vimentin and fetal-type intermediate filament cytokeratins (CK) 8 and 18 [23,36,37,38]. We also demonstrated that EMT of FIB and NuB1 cells involved increased expression of myofibroblast proteins, namely, vimentin, alpha-smooth muscle actin as well as muscle and non-muscle caldesmon [46]. Hence, neoplastic transformation of immortalized mucosal gingival epithelial cells involves epithelial myofibroblast transition to be included into the ‘EMT’ definition. We further reported that cellular contractility of spindle FIB and NuB1 cells was increased compared with that of cobblestone cells [46]. Myocyte enhancing factor 2B (MEF2B) is the only MEF2 isoform which is expressed during early embryonic development, and it may transactivate downstream target EMT proteins. Expression of MEF2B mRNA and myofibroblast proteins was higher in spindle NuB1 and FIB cells compared to cobblestone cells. EGF treatment of cobblestone control cells also induced expression of these genes. Knockdown of MEF2B in a cobblestone cell line abolished EGF-induced upregulation of MEF2, vimentin and non-muscle caldesmon proteins [37]. Thus, our work provides an additional mechanism that EMT of immortalized gingival epithelial cells may involve activation of MEF2B associated with myogenic phenotype.

ROS are known to regulate proteins that are important for degradation of extracellular matrices, such as, gelatinase matrix metalloproteinases (MMP)-2, -9, which are key enzymes for degrading type IV collagen and thought to play a critical role in tumor invasion and metastasis. We showed that EPI cells secreted latent MMP-9 (92 kDa) and MMP-2 (72 kDa) [47]. FIB cells released a protein at 86 kDa with a slight lower molecular weight than latent MMP-9 [45,47]. MMP-9 at 86 kDa is known as an intermediate molecule that regulates CK18 at post-translational level in Nox1-overexpressing cells. We here show new results: treatment of NuB2 cells with a PKC inhibitor G06983 decreased expression of CK18 protein in cells alone or upon activation with PKC activator PMA (Fig. 3A). G06983 or PKCepsilon siRNA concomitantly inhibited EGF-induced ROS generation (Fig. 3B). Transfection of NuB2 cells with PKCepsilon siRNAs decreased expression of CK18 protein when compared with control siRNA (Fig. 3C). We therefore provide new evidence that PKCepsilon could be a mediator of Nox1 and ROS which regulate a post-translational accumulation of CK18.

Role of PKCepsilon in CK18 accumulation by Nox1

It has been known that NADPH oxidase enzymes can be activated by beta-, delta- and epsilon PKC isotypes [52]. In this context, PKCepsilon has been shown to associate with and phosphorylate CK18 [53], and thus PKCepsilon was proposed to be a signaling molecule that regulates CK18 at post-translational level in Nox1-expressing cells. We here show new results: treatment of NuB2 cells with a PKC inhibitor G06983 decreased expression of CK18 protein in cells alone or upon activation with PKC activator PMA (Fig. 3A). G06983 or PKCepsilon siRNA concomitantly inhibited EGF-induced ROS generation (Fig. 3B). Transfection of NuB2 cells with PKCepsilon siRNAs decreased expression of CK18 protein when compared with control siRNA (Fig. 3C). We therefore provide new evidence that PKCepsilon could be a mediator of Nox1 and ROS which regulate a post-translational accumulation of CK18.

Thus, our data showed that CK18 phosphorylation at ser-52 was dependent on Nox1, PKC and ROS. These events may lead to cytoskeletal dynamics associated with the reorganization of keratin filaments in cell division. In Nox1-expressing cells, PKCepsilon may be a key kinase as it is able to phosphorylate CK18 [53]. As depicted in Fig. 3D, ligation of EGF tyrosine kinase receptor during cell growth activates PKCepsilon leading to Nox1 activation and oxidant generation. PKCepsilon alone or Nox1-mediated oxidants may induce phosphorylation of CK18. This prevents CK18 from being ubiquitinated resulting in decreased Ub-CK18 thereby increased CK18 accumulation. The use of DPI, apocynin, NAC, G06983, and PKCepsilon siRNA was able
to reverse the processes resulting in decreased CK18 protein levels. Phosphorylation of CK18 at ser-52 by PKCepsilon-mediated Nox1 activation may be an important key step in determining the levels of CK18 subjected for ubiquination (Fig. 3D). PKCepsilon is shown to increase cell growth and inhibits apoptosis [54] in human HaCaT keratinocytes which express Nox1. While Nox1 is activated by Ras in human colon cancer, PKCepsilon has also been shown to interact with ras signaling pathway in colon epithelial cells [55]. PKCepsilon has been shown to advance progression of human prostate cancer [56], which highly expresses Nox1. PKCepsilon not only regulates CK18 as demonstrated in our studies, it also regulates vimentin [57] and actin [58] in the control of cell motility and fiber organization.

PKCepsilon may be considered an effector molecule of Nox-based enzymes, particularly Nox1, in promoting transformation progression of and cancer cells for CK18 stabilization in a PKCepsilon-, ROS-, and phosphorylation-dependent manner. This post-translational mechanism for CK18 accumulation is consistent with the persistent presence of CK18 in many epithelial carcinomas. Our data also indicate that PKCepsilon is an important mediator that contributes to CK18 persistence, proliferation and apoptosis resistance in Nox1-expressing cells. Nox1 and PKCepsilon may thus be used as targets to inhibit the progression of preneoplastic and cancer cells.

Role of Nox1 in accelerating transformation beyond immortalization steps

To study the role of Nox1 and Nox1-derived ROS in human epithelial cancers, we accomplished our goals in establishing preneoplastic model cells from HPV16-immortalized gingival mucosal cells by chronic ethanol or by Nox1 overexpression. Phenotypic characterization of resultant cell lines indicates progressive steps in cell transformation with spindle cells at higher steps than cobblestone epithelial cells. Spindle cells associated with EMT are likely more migratory and invasive. Molecular targets of Nox1 involve p16INK4a, survivin, MMP, MEF2B, CK18, and PKCepsilon, while the latter three regulate EMT. Taken together, Nox1 mediates the progression of immortalized human epithelial cells towards tumorigenicity partly by an induction of EMT. In human epithelial cancers,
Nox1 may be functional together with other oncogenes in accelerating neoplastic progression.

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A. Sattayakhom et al. / Redox Biology 2 (2014) 140 – 147

146

A. Sattayakhom et al. / Redox Biology 2 (2014) 140 – 147

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