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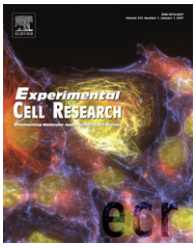

Research Article

Duox1 is the main source of hydrogen peroxide in the rat thyroid cell line PCCl3

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ABSTRACT

Duox1 and Duox2 proteins are particular members of the NADPH oxidase (Nox) family and were first characterized as the thyroid NADPH oxidases. These proteins are responsible for the hydrogen peroxide (H_2O_2) production necessary for the synthesis of thyroid hormones. Although mutations in the *Duox2* gene have been discovered in hypothyroid patients with iodide organification defects, attempts to confirm the role of one or both proteins in the generation of H_2O_2 in the thyroid were unfruitful. Using the RNA interference technique, we demonstrated in this study that Duox1 is the main source of H_2O_2 in the rat thyroid cell line PCCl3. We showed that (1) Duox1 was abundantly expressed in PCCl3 in regard to Duox2, contrary to what was observed in the rat thyroid tissue; (2) the expression of a siRNA specifically targeting Duox1-induced silencing of its transcript and the corresponding protein with a parallel decrease of H_2O_2 production; (3) the re-expression of Duox1 in silenced cells by a lentivirus based method rescued totally H_2O_2 production with rat Duox1 and partially with human Duox1. Western blotting analysis confirmed the synthesis of the mature N-linked glycosylated protein responsible for this enzymatic activity.

Introduction

Thyroid hormone synthesis is a multistep process starting with the uptake and the concentration of iodide which is oxidized before its binding to specific tyrosyl residues of the thyroglobulin (Tg) in the follicular lumen. Iodide is actively transported through the basal membrane by the Na^+/I^- symporter (NIS) [1–3] and leaked out or pumped out at the apical membrane along the electric gradient through an iodide channel, which could be pendrin [4–6]. In the follicular lumen, iodide is organified by the thyroperoxidase (TPO) [7] using hydrogen peroxide (H_2O_2) as cofactor which constitutes the limiting step of the hormonogenesis [8]. It has long been known that the thyroid H_2O_2-generating system is a NADPH oxidase. This enzyme seems to produce H_2O_2 directly rather than via initial production of superoxide anions [9–11]. Duox1 and Duox2 have been isolated and characterized first as the thyroid oxidases [12,13]. Human Duox1 and Duox2 are very

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Abbreviations: Tg, thyroglobulin; TPO, thyroperoxidase; NIS, Na^+/I^- symporter; rDuox, rat Duox; hDuox, human Duox; DuoxA, Duox Activator; ER, endoplasmic reticulum; NADPH, nicotamide adenine dinucleotide phosphate; siRNA, small interfering RNA; TSH, thyroid-stimulating hormone

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similar proteins displaying 83% sequence similarity. They are co-localized with TPO at the apical membrane of the thyrocyte. They present the characteristics of heme bound NADPH oxidases (Nox) like Nox1–Nox5 but with two EF-hand sequences in the first intracellular loop most probably conferring their sensitivity to Ca^{2+} and an extracellular NH_2 terminus peroxidase-like domain. They present two glycosylation states shifting the molecular mass from 160 to 180 and 190 kDa. The 190 kDa forms presumably correspond to the mature proteins correctly processed at the plasma membrane [14].

Duox1 and Duox2 are expressed in other organs than the thyroid, for example, in the prostate [15] and in the respiratory tract epithelium [16]. In addition, Duox2 has been found in salivary glands, in rectal mucosa and all along the digestive tract, mostly in the colon, where it could be implicated in the innate immunity [17–19]. Duox1 and Duox2 mRNA are highly expressed in the thyroid. In human thyroid, Duox2 mRNA is 1.5–5 times more abundant than Duox1 mRNA as determined by northern blotting [20] or by SAGE [21], and both transcripts remain expressed at the same order of magnitude in toxic adenomas, hypofunctioning nodules or in Graves' disease [22–25]. The respective roles of Duox1 and Duox2 proteins in the thyroid are not yet totally elucidated. However mutations in *Duox2* gene have been described for patients suffering from congenital hypothyroidism with organification defect not due to *TPO* mutations [26–28]. These mutations cause early stop codons leading to truncated proteins without NADPH oxidase catalytic site. These results prove, at least in human thyroid, that Duox2 is necessary to provide H_2O_2 for the synthesis of thyroid hormones and that the defect of H_2O_2 production due to mutated Duox2 is not compensated by Duox1 in that tissue.

Until recently, we had no proof of a direct involvement of Duox isozymes in the production of H_2O_2 because transfections of Duox1 and/or Duox2 cDNAs lead to the translation of an incompletely processed protein blocked in the endoplasmic reticulum (ER) compartment without any functional activity [14]. Duox activator (DuoxA2) has been identified as a maturation factor allowing the transition of the glycosylated Duox2 from ER to Golgi and its functional expression at the plasma membrane in heterologous systems [29].

In this work, we investigated the role of rat Duox proteins (rDuox) in PCCl3, a rat thyrocyte cell line with a functional H_2O_2-generating system [14,30]. We used the RNA interference technique in order to measure H_2O_2 production when Duox1 or Duox2 expressions were selectively silenced. We demonstrate that rDuox1, which is the main expressed isoform in PCCl3 cells, is responsible for the Ca^{2+}-dependent H_2O_2 production.

Materials and methods

Design and construction of siRNA expression plasmids

pGE-1 from Stratagene, containing the human U6 RNA polymerase III promoter and the neomycin/kanamycin-resistance genes, was used for the expression of siRNA (small interfering RNA). The 19-nt sequences corresponding to siRNAs targeting rat Duox1 or rat Duox2 (rDuox1 or rDuox2) were designed with the siRNA sequence selector of Clontech (http://bioinfo.clontech.com/rnaidesigner/). At least three oligonucleotides bearing 100% sequence identity with rDuox1 or rDuox2 cDNA sequences were chosen after a blast homology search to confirm their sequence specificity. Their position relative to the start ATG in Duox1 and Duox2 is represented in Table 1. The structure of the insert is the following: *Bam*HI cloning site, the 19-nt oligonucleotide (sense), a 9-nt sequence corresponding to the hairpin loop, the reverse complementary 19-nt sequence (antisense), a transcription terminator sequence of 6-nt poly(T). The complementary strand was 5′-ended with *Eco*RI cloning site. Oligonucleotides were synthesized by Eurogentec. After cloning in pGE-1 vector by *Bam*HI and *Eco*RI digestions, purified DNA plasmids were verified by *Xho*I/*Xba*I restrictions and the sequences were checked by Big Dye Terminator cycle sequencing on an automated ABI Prism 3100 sequencer (Applied Biosystem).

H_2O_2 measurement

H_2O_2 measurements were performed according to the method of Bénard and Brault [31]. PCCl3 cells were incubated in Krebs–Ringer HEPES (KRH) medium pH 7.4 containing 0.1 μg/ml horseradish peroxidase type II (Sigma), 440 μM homovanillic acid (Sigma) and 1 μM ionomycin (Sigma) which allows the rise of intracellular Ca^{2+} concentration. Fluorescence intensity of oxidized homovanillic acid was measured at an emission wavelength of 425 nm after excitation at 315 nm using a fluorimeter LS50B (Perkin-Elmer). H_2O_2 concentrations were determined according to a standard curve with known H_2O_2 concentrations incubated in the same conditions. The cells were washed with PBS, lyzed in Laemmli buffer and protein content was measured by a paper dye binding assay [32] to normalize H_2O_2 measurements.

Cell transfection

PCCl3 cells (Coon's modified Ham's F-12 medium (Invitrogen) with 5% decomplemented fetal bovine serum, 5 μg/ml transferrin, 1 mU/ml bovine TSH, 1 μg/ml insulin, 2% penicillin–streptomycin, 1% fungizone) were transfected with siRNA constructs (pGE1–siRNA) using FuGENE6 reagent according to the manufacture's protocol (Roche Molecular Biochemicals). A selection of 4 days with 400 μg/ml neomycin was performed for transient transfection. Stable cell lines expressing the siRNA were established by limit dilution and selection with 400 μg/ml neomycin. Co-transfections of CHO cells (Ham's F-12, 10% fetal bovine serum, 2% streptomycin–penicillin, 1% fungizone, 1% sodium pyruvate) with rDuox1 or rDuox2 in pcDNA3.0 (200 ng) (Invitrogen) and pGE1–siRNA (500 ng) were performed with FuGENE6 for 72 h.

Production of lentivirus expressing Duox1 and in vitro transduction

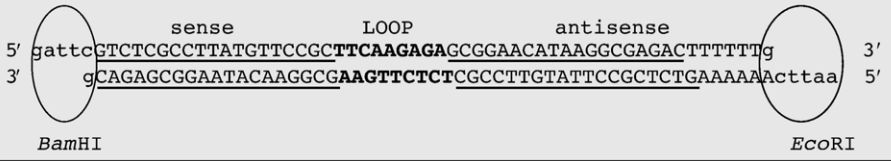
The open reading frames encoding rDuox1 and human Duox1 (hDuox1) were cloned (5′ *Mlu*I – 3′ *Eco*RI) into pWXLd-GFP lentiviral vector kindly given by D. Trono (School of Life Sciences, Lausanne, Switzerland) in place of GFP cDNA. All

Table 1 – (A) Name and position from start ATG of oligonucleotide sequences in rDuox1 and rDuox2 cDNA encoding siRNAs; (B) Structure of the double strand oligonucleotide cloned in the pGE-1 vector encoding a siRNA

A

		Position from start ATG
Rat Duox1		
D1-44	5′-CATCAGGGCAGCTCTGAAG-3′	2184
D1-65	5′-GATCTGCCCTTCACCTAGA-3′	2883
D1-67	5′-ACAATACGAAGACAGCGTC-3′	2924
D1-72	5′-CTTCATTCCAGCCCTTACT-3′	3020
Rat Duox2		
D2-30	5′-GATCTGAGAGATTACTGGC-3′	1225
D2-31	5′-GAATTGGAGTGCTCTCAAC-3′	1362
D2-43	5′-CCGAGAACGCAAGATGCTA-3′	1872
D2-46	5′-AGAAAGGCAAGGAGAGTCT-3′	1895
D2-70	5′-GGAGGAATTCTTCACCATG-3′	2631
D2-90	5′-CGTGTACATCTTCTCAGTC-3′	3351
Loop	5′-TTCAAGAGA-3′	

B



the constructions were verified by sequencing. Lentiviral vectors were produced by transient transfection into Hek293FT cells (Invitrogen) using the calcium phosphate method in a biosafety level 2 environment. Briefly, 2.5×10^6 cells were seeded in 10-cm diameter dishes 24 h prior to transfection in DMEM (Invitrogen) containing 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum and 1% penicillin–streptomycin. The precipitate was formed by mixing 20 μg of the transfer vector (pWXLd), 15 μg of the packaging plasmid (psPAX2) and 6 μg of the envelope plasmid (pMD2G) to a final volume of 500 μl water containing 250 mM $CaCl_2$, and adding this mix dropwise into 500 μl of 2× HEPES-buffer saline (280 mM NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$ pH 7.12). The psPAX2 and pMD2G plasmids were also provided from D. Trono. The conditioned medium was collected after 48 h, cleared by low-speed centrifugation and filtered through 0.45 μm low protein binding filter. For PCCl3 transduction, viral stocks were concentrated by ultracentrifugation. The pWXLd-GFP virus stock was titrated by serial dilution on PCCl3 cells in the presence of polybrene (3 μg/ml) followed by FACS analysis (FACScan, Becton Dickinson). The titer of the pWXLd-rDuox1 and pWXLd-hDuox1 virus was estimated from the p24 antigen concentration determined with an enzyme immunoassay (Innotest HIV antigen mAb, Innogenetics). For PCCl3 transduction, 50×10^3 cells were infected by the viral particles during 24 h at a multiplicity of infection (MOI) of 10 in the presence of polybrene (3 μg/ml).

Western blot analysis

Thirty μg of total protein extracts from PCCl3 lyzed in Laemmli buffer were separated by SDS/polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose as previously described [12]. Duox proteins were detected using a rabbit polyclonal antibody raised against the first intracellular loop of human Duox1 (I2) [12] at a dilution of 1/8000 or a rabbit polyclonal antibody raised against the first intracellular loop of human Duox2 (F2) [33] at a dilution of 1/5000 kindly provided by C. Dupuy (IGR, Villejuif, France). Actin was detected by a polyclonal antibody (Sigma) at a dilution of 1/750 to verify the amount of loaded proteins. The immune complexes were detected by horseradish peroxidase-conjugated antibodies according to the ECL method (Perkin-Elmer Life Sciences).

Total RNA isolation and semi-quantitative RT–PCR

Total RNA was extracted from transfected PCCl3 cells with Trizol (Invitrogen) and purified on RNeasy columns (Qiagen). After reverse transcription, the cDNAs were amplified by *Taq* polymerase (Invitrogen) using the following primers: rat-Duox1 forward 5′-CCACTGAAGTTTTCCCGTACA-3′ and reverse 5′-CCTGCAAGCCAAAAGAAGAC-3′ (26 cycles), rat-Duox2 forward 5′-CGCATAGCTGAGATGGATGA-3′ and reverse 5′-AGAGGGAGCCATTACCCTGT-3′ (30 cycles), GAPDH forward 5′-GTCAACGGATTTGGTCGTAT-3′ and reverse 5′-CACTGC-CACCCAGAAGACTG-3′ (23 cycles), rat-NIS forward 5′-GTGG-CATTGTCATGTTCGTC-3′ and reverse 5′-CGTGAAGGCGC-CTAGTAGAG-3′ (23 cycles). The PCR conditions were 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min. PCR products were visualized on a 1% agarose gel. For the detection of Duox mRNAs in rat thyrocyte cell lines and in rat thyroid tissue (Sprague Dawley rat strain), cDNAs were PCR amplified with 35 cycles at 95 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min using the following primers common for rDuox1 and rDuox2: rat-Duox1–2A forward 5′-GACTTCCATTTCATGCTGCG-3′ and

reverse 5′-AGGTGATGAGGTTGCGGCAC-3′. The regions amplified for rDuox1 and rDuox2 are: 2747–3331 and 2759–3229, respectively. After DNA staining with ethidium bromide, the agarose gel was scanned with a Typhoon Trio+ (Amersham Biosciences).

Subcloning of rDuox1 and rDuox2 from PCCl3 and rat thyroid tissue

cDNA of PCCl3 and rat thyroid tissue was amplified (40 cycles at 95 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min) using the primers rat-Duox1-2B forward 5′-ATCATCTAGAAGCAGGTCAACCTCATCCTG-3′ including a *Xba*I restriction site and rat-Duox1-2B reverse 5′-TGACCTCGAGGAAATGGAAGTCCTCCCAGG-3′ including a *Xho*I restriction site. The 710 pb PCR product band was cut from agarose gel 1% and extracted using MinElute gel extraction kit (Qiagen). The purified products were restricted by *Xho*I and *Xba*I, subcloned into pBluescript Sk+ vector and transformed in DH10B *Escherichia coli*. Colonies were screened by DNA restriction using *Xba*I/*Stu*I and *Xho*I/*Nco*I. The plasmids were sequenced using the T3 universal primer.

Iodide uptake

PCCl3 cells were preincubated in 1 ml of KRH for 30 min followed by 1 h incubation in the same medium containing 1 mCi ^{125}I and 10^{-7} M KI. At the end of the incubation, the medium was removed and the cells were quickly rinsed with PBS before lysis in 1 ml 1 N NaOH. Cells were incubated in parallel with 10^{-4} M $NaClO_4$ to block iodide entry. Radioactivity was measured in a gamma Wizard Counter (Perkin-Elmer). Results were normalized to protein concentrations.

Results

Efficiency and specificity of the siRNAs targeting rDuox1 and rDuox2 tested in a heterologous system

The anti-Duox antibodies raised against Duox1 (I2) [12] and Duox2 (F2) [33] are not able to distinguish the two isoforms. Until now, there is no serum able to discriminate Duox1 from Duox2. Before using our siRNA constructs on the PCCl3 cell line, we have tested their specificity on CHO cells expressing one or the other Duox isoform (rDuox1-pcDNA3.0 and rDuox2-pcDNA3.0). Duox1 and Duox2 proteins separately over-expressed in CHO cells, are visualized with the antibodies as one immunoreactive band corresponding to the immature high mannose form of Duox at a molecular mass of 150 kDa [14] (Fig. 1). The Neg siRNA construct, provided by Stratagene, was used as negative control of RNA interference: it is constituted of the pGE-1 vector coding for a siRNA that does not suppress expression of any gene expressed in human, rat or mouse. The Neg siRNA construct had no effect on rDuox1 or rDuox2 protein expression detected with polyclonal antibodies I2 or F2, respectively (Fig. 1A). Four siRNA constructs targeting specifically rDuox1 transcript (D1-44, D1-65, D1-67, D1-72) provoked a reduction of the expression of the corresponding protein and have no effect on rDuox2 expression (Fig. 1B). The six constructs encoding siRNAs against rDuox2 (D2-30, D2-31, D2-43, D2-46, D2-70, D2-90) were efficient in selectively silencing rDuox2 expression with no effect on rDuox1 protein level (Fig. 1C). D2-70 and D2-90 inhibited almost total rDuox2 expression. These results showed that the designed siRNAs allow specific silencing of rDuox1 and rDuox2 protein expression in the CHO cell line.

Transient expression of rDuox siRNA in PCCl3 cells

PCCl3 is a rat thyrocyte cell line often used as a thyroid model [30]. PCCl3 cells possess a functional H_2O_2-generating system sensitive to Ca^{2+} stimulation. In basal conditions, these cells produce a small quantity of H_2O_2 which is strongly increased after treatment with the Ca^{2+} ionophore, ionomycin [14] (Fig. 2A). To analyze the respective role of rDuox1 and rDuox2 in H_2O_2 generation, PCCl3 were transiently transfected with the siRNA constructs previously tested in co-transfection experiments in the CHO cells. The effect of these siRNAs was tested on H_2O_2 production after treatment with ionomycin. The production of H_2O_2 was not significantly affected by the expression of Neg siRNA and was therefore considered as the 100% activity value in most of the experiments (Fig. 2A). PCCl3 cells expressing siRNAs targeting rDuox1 transcript produced 50% of the amount of H_2O_2 generated by PCCl3 cells trans-

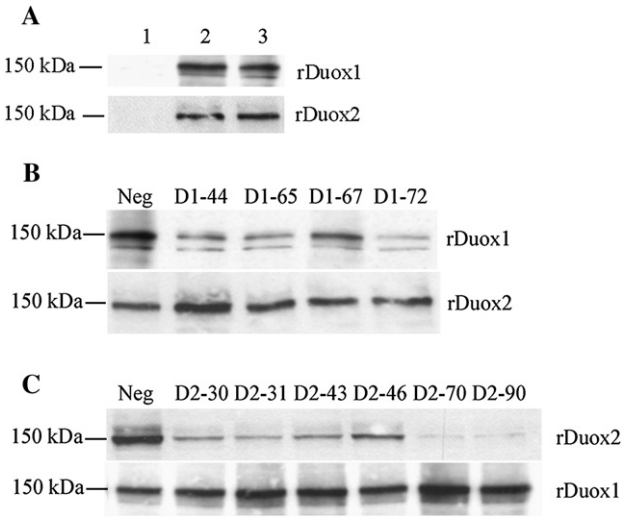


Fig. 1 – Efficiency and specificity of the rDuox siRNAs in the CHO heterologous system. CHO cells were co-transfected with rDuox1-pcDNA3.0 or rDuox2-pcDNA3.0 and with pGE-1 constructs encoding siRNAs. 10 μg of total proteins were equally loaded and resolved by SDS/PAGE electrophoresis. rDuox1 protein expression was immunodetected with anti-Duox I2 antibodies (dilution 1/8000) and rDuox2 was immunodetected with the F2 antibodies (dilution 1/5000). (A) rDuox expressions in CHO co-transfected with negative control siRNA (Neg). 1: pcDNA3.0 alone, 2: rDuox1 or rDuox2-pcDNA3.0. 3: rDuox1 or rDuox2-pcDNA3.0 + Neg siRNA. (B) Efficiency and specificity of the siRNAs targeting rDuox1. (C) Efficiency and specificity of siRNAs targeting rDuox2.

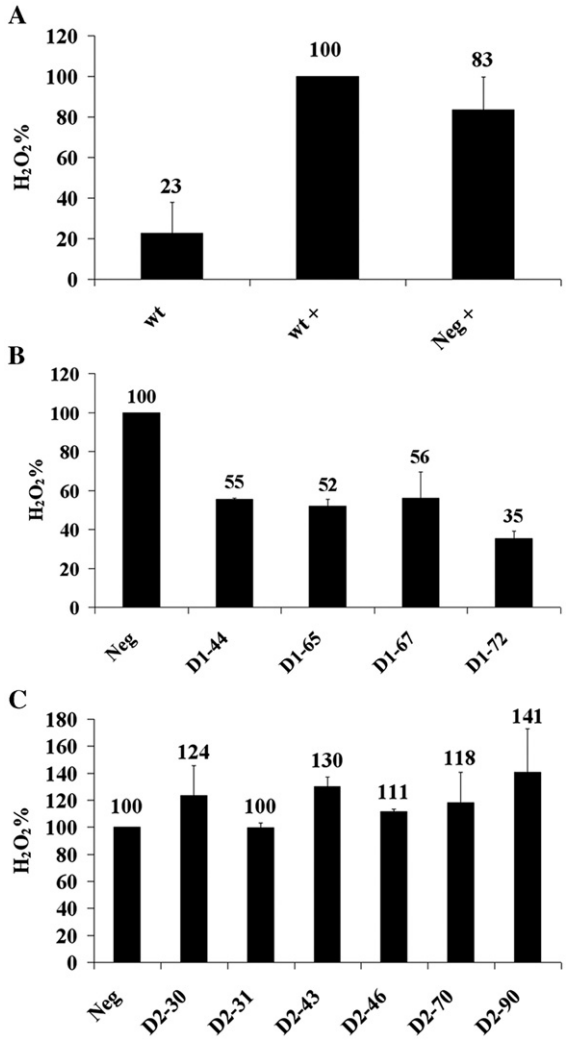


Fig. 2 – H_2O_2 production by PCCl3 transiently expressing a siRNA interfering with rDuox1 or rDuox2. H_2O_2 production by PCCl3 transfected with different constructs encoding siRNAs was measured during 1 h and 30 min at 37 °C as described in Materials and methods. Each sample was tested in duplicate and the H_2O_2 expressed in ng/μg protein was represented in percentage of the value of H_2O_2 obtained with wild type PCCl3 (wt) or PCCl3 transfected with the Neg siRNA treated with ionomycin. (A) Wild type PCCl3 (wt) and PCCl3 transfected with Neg siRNA were treated (+) or not by ionomycin 1 μM. (B) Ionomycin stimulated PCCl3-expressing Neg siRNA or 4 different rDuox1 siRNAs (D1-44, D1-65, D1-67, D1-72). (C) H_2O_2 generation by PCCl3-expressing Neg siRNA or 6 different rDuox2 siRNAs (D2-30, D2-31, D2-43, D2-46, D2-70, D2-90) and stimulated with ionomycin. These experiments are representative of two independent experiments.

fected with Neg siRNA plasmids (Fig. 2B). However, PCCl3 cells transfected with six different rDuox2 siRNA constructs did not show any significant decrease in their H_2O_2 production (Fig. 2C). As the two antibodies are not able to discriminate the two Duox isoenzymes, the silencing effect of the siRNA was evaluated on their mRNA expression by conventional RT–PCR using specific primers for each transcript. rDuox1 mRNA level (visualized after 26 cycles of PCR on reverse transcribed mRNA) was decreased in PCCl3 expressing siRNAs which target rDuox1 (Fig. 3A). The expression of rDuox2 transcript (visualized after 30 cycles of PCR) was not affected by the co-expression of the six different siRNA constructs targeting rDuox2 (Fig. 3B). These results showed a perfect correlation between silencing of endogenous rDuox1 expression and the decrease of H_2O_2 generation, suggesting that rDuox1 is the relevant source of H_2O_2 in these cells and showing that rDuox2, expressed at very low level in PCCl3, little contributes to H_2O_2 synthesis.

Since the two Duox mRNAs have been found in the thyroid of most of the species (human, dog, rat, pig, etc.) [12–14,34], we determined the relative levels of Duox1 and Duox2 transcripts in PCCl3 cells by PCR amplification and sequencing. A couple of primers (rat Duox1–2A forward and reverse) was designed to anneal identical regions in rDuox1 and rDuox2 but amplifying sequences of different sizes (580 bp for rDuox1 and 470 bp for rDuox2). rDuox1-pcDNA3.0 and rDuox2-pcDNA3.0 plasmids were PCR amplified with the same primers to control similar amplification efficiency for the two Duox cDNAs (Fig. 4A). RT–PCR was performed on total RNA from three rat thyroid cell lines: PCCl3, FRTL5 and WRT and on total RNA from rat thyroid tissue (Fig. 4B). In these three thyroid cell lines, the expression of rDuox1 mRNA was far more important (~80% of total Duox transcripts) than the expression of rDuox2 (~20%) as estimated by quantification of the intensity of the amplified fragment. In contrast, the rDuox2 mRNA expression was slightly higher (~57%) than rDuox1 (~43%) in the rat thyroid tissue (Fig. 4C). The relative expression of Duox1 and Duox2 mRNA in the wild type PCCl3 and rat thyroid tissue was confirmed by RT–PCR using primers rat-Duox1-2B (see Materials and methods) followed by subcloning, restriction and sequencing of the PCR products. Starting from PCCl3 total RNA, 87% of the analyzed colonies possessed the Duox1 plasmid and 13% the Duox2 plasmid; for rat thyroid tissue, we obtained 43.5% of colonies containing Duox1 versus 56.5% containing Duox2.

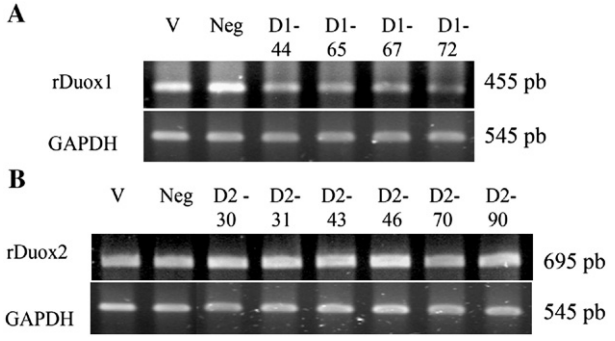


Fig. 3 – Silencing of rDuox1 and rDuox2 mRNA expression in PCCl3 transiently transfected with siRNA constructs. Total RNA extracted from transfected PCCl3 cells with Neg, rDuox1 or rDuox2 siRNA constructs was reverse transcribed and PCR amplified using specific primer for rDuox1 (A) or rDuox2 (B). GAPDH was amplified to estimate an equal efficiency of amplification in each sample. V: PCCl3 transfected with the pGE-1 vector alone.

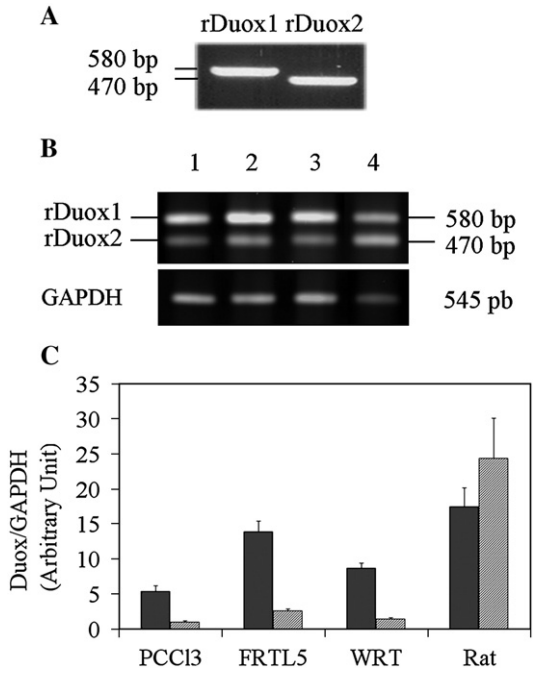


Fig. 4 – Expression of rDuox transcripts in PCCl3, FRTL5, WRT and rat thyroid tissue by RT–PCR. (A) PCR amplification of 1 pM rDuox1-pcDNA3.0 or rDuox2-pcDNA3.0 cDNA using primers common to Duox1 and Duox2 (primers rat-Duox1-2A forward and reverse). These primers amplify a fragment of 580 bp from rDuox1 and a fragment of 470 bp from rDuox2. (B) Amplification of Duox1 and Duox2 cDNAs obtained by reverse transcription of total RNA from PCCl3 (1), FRTL5 (2), WRT (3) and rat thyroid tissue (4) with the same primers as in panel A. GAPDH amplification was used for normalization. These results are representative of four independent experiments. (C) Relative densitometry of Duox1 (black) and Duox2 (grey) expression; summarized data from four independent experiments.

Stable silencing of rDuox1 in PCCl3

As PCCl3 mainly expressed rDuox1 and siRNAs against rDuox2 had no silencing effect, we focused our study on rDuox1. D1-65 and D1-72 siRNA constructs, which produced the most important decrease of H_2O_2 production in transient transfection experiments (Fig. 2B), were transfected as well as the Neg siRNA, in PCCl3 to establish stable expressing cell lines. Ten clones expressing rDuox1 siRNA were selected for each siRNA constructs after limit dilution, four of them are described in Fig. 5. Silencing of endogenous rDuox1 protein expression was analyzed by Western blotting using the I2 anti-Duox antibody. Detection of actin was used as loading control. Compared to wild type PCCl3, Neg PCCl3 clones did not show any significant decrease of rDuox protein expression (Fig. 5A). Two N-linked glycosylated forms of rDuox protein were detected. They correspond to the mature (160 kDa) and immature (150 kDa) proteins [14]. D1-65#1, D1-65#3, D1-72#3 and D1-72#9 siRNA clones showed the most important decrease of rDuox protein expression (Fig. 5A). The silencing effect of these siRNAs was also observed at the level of rDuox1 mRNA synthesis with no interference on the rDuox2 mRNA level (Fig. 5B).

H_2O_2 production was examined in the PCCl3 clones treated with ionomycin. Six independent clones expressing the Neg siRNA produced amounts of H_2O_2 similar to wild type PCCl3, confirming that this siRNA did not interfere with rDuox expression (Fig. 6A). Most of the PCCl3 clones, in which rDuox1 was silenced, produced less H_2O_2 than the Neg siRNA cells (Figs. 6B–C); this inhibition was clearly correlated with lower levels of rDuox1 protein (Fig. 5A).

The main function of the thyroid is to concentrate and organify iodide to synthesize thyroid hormones. In order to exclude an overall nonspecific effect of silencing which could influence the thyrocyte function, iodide uptake capacity was analyzed in D1-65 PCCl3 cells. NIS mRNA level estimated by RT–PCR remained unchanged and was comparable to the level in wild type PCCl3 or in PCCl3-expressing Neg siRNA (Fig. 7A). Moreover, iodide uptake, which is abolished by the NIS competitive inhibitor $NaClO_4$, was not affected in the D1-65 PCCl3 clones and was similar to the uptake in the Neg siRNA

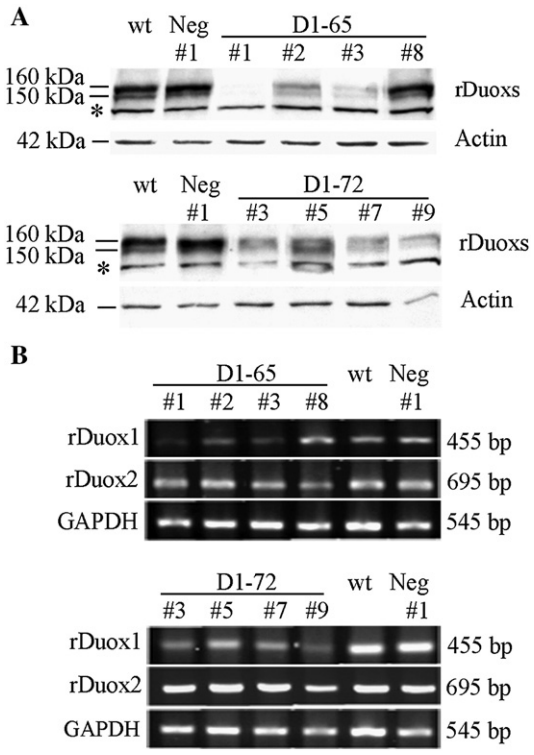


Fig. 5 – Silencing of rDuox1 in PCCl3 clones expressing the D1-65 and D1-72 siRNAs. (A) Duox expression in PCCl3 clones stably transfected with D1-65 and D1-72 siRNA constructs. 4 out of 10 representative clones are shown. 30 μg of total protein extracts were loaded on a 6% SDS–PAGE gel. rDuox proteins were immunodetected with the I2 antibody at a dilution of 1/8000. Actin (antibody from Sigma, dilution 1/750) was immunodetected as control of protein loading. (*): non-specific band. (B) rDuox transcripts expression measured by RT–PCR on total RNA from wild type PCCl3, Neg, D1-65 or D1-72 PCCl3 clones using specific primers for rDuox1 (455 bp), rDuox2 (695 bp) and GAPDH (545 bp) for normalization.

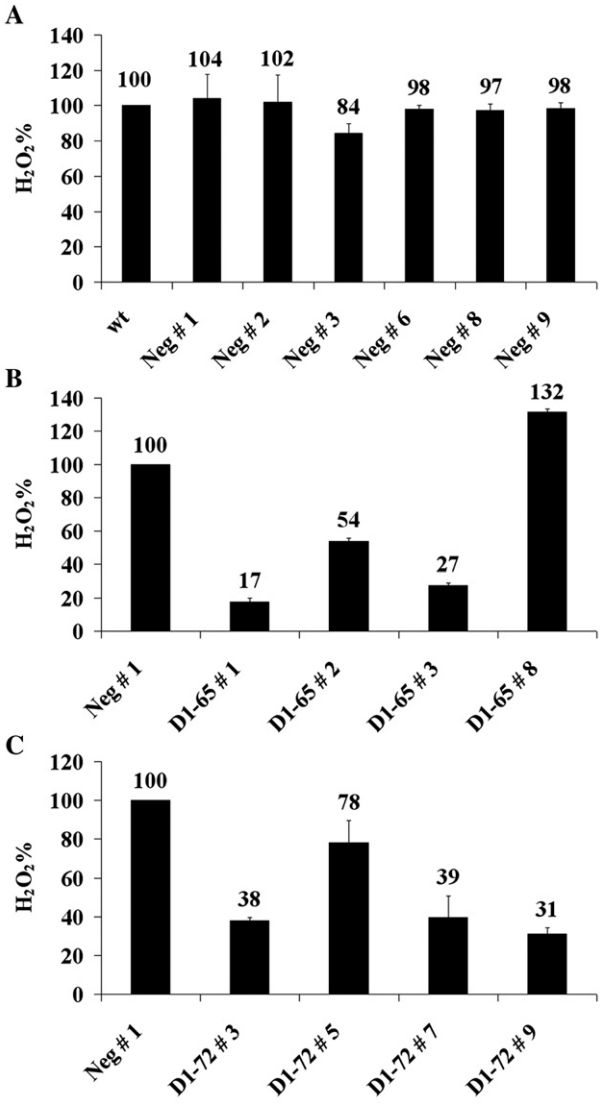


Fig. 6 – H_2O_2 production by PCCl3 clones expressing D1-65 or D1-72 siRNAs. PCCl3 cells were stimulated by 1 μM ionomycin 1 h and 30 min at 37 °C and the H_2O_2 production was measured as described in Materials and methods. Each sample was tested in duplicate. (A) H_2O_2 production from wild type PCCl3 corresponding to 100% activity and 6 individual clones expressing the Neg siRNA. (B) H_2O_2 production from Neg#1 PCCl3 corresponding to 100% activity and 4 clones expressing the D1-65 siRNA. (C) H_2O_2 production by Neg#1 PCCl3 and 4 clones expressing the D1-72 siRNA.

clone (Fig. 7B). These results confirm that the D1-65 siRNA specifically targets rDuox1 without interfering with the thyroid phenotype of the PCCl3 thyroid cell line.

Lentivirus-based over-expression of Duox1 in D1-65 PCCl3

To demonstrate that Duox1 is able to produce H_2O_2, we over-expressed the protein in two independent PCCl3 clones, D1-65#1 and D1-65#3, characterized by a very low expression level of endogenous rDuox1 and a low H_2O_2 production. Vesicular stomatitis virus (VSV) G-pseudotyped lentivirus particles are able to mediate efficient delivery, integration and stable expression of transgenes in a broad type of cells, especially in cells, like PCCl3, which are poorly transfected by classical transfection methods [35,36]. Using the pWXLd lentiviral vector, from Trono's lab, expressing the GFP protein (pWXLd-GFP), we have shown that 25×10^4 infectious particles were needed to efficiently transduce one PCCl3 cell suggesting that this cell line is very resistant to the virus infection. Nevertheless, using a multiplicity of infection (MOI) of 10, more than 90% of PCCl3 cells were transduced (data not shown). After infection of D1-65#1 and D1-65#3 cells with the lentivirus pWXLd-GFP, pWXLd-rD1 or pWXLd-hD1, H_2O_2 production of the transduced cells was measured in the presence or not of ionomycin (Fig. 8A). Lentiviral particles expressing GFP did not significantly modify the H_2O_2 production activity of D1-65#1 (14%) and D1-65#3 (45%) compared to the non-infected cells (15% and 39% respectively). In contrast, over-expression of rDuox1 rescued H_2O_2 generation in the two clones at a level close to the wild type PCCl3 (138% and 166% respectively). Over-expression of hDuox1 increased also H_2O_2 production (42% vs. 14% and 83% vs. 45%) but with a lower potency than rDuox1 protein. These results provide evidence that human and rat Duox1 are functional NADPH oxidases able to produce H_2O_2. Western blot analyses confirmed Duox over-expression in the transduced cells (Fig. 8B, upper panel) and showed the protein as a doublet with the high molecular weight form corresponding to the active maturated protein, contrary to

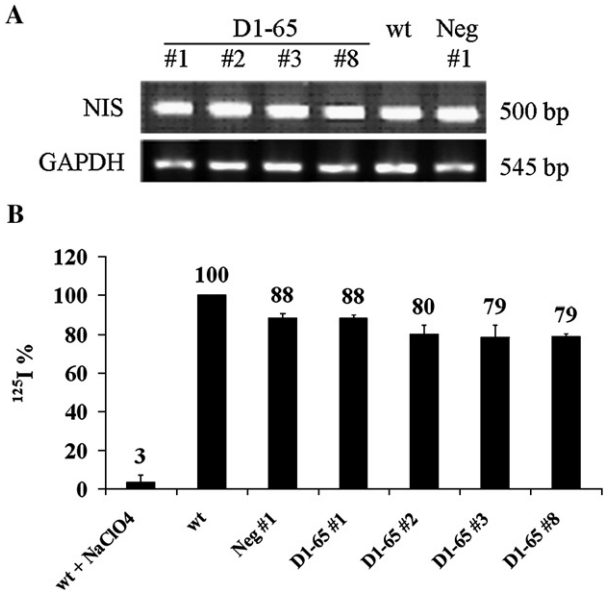


Fig. 7 – Expression of NIS and iodide uptake in PCCl3 expressing D1-65 siRNA. (A) Expression of NIS mRNA by RT–PCR on total RNA isolated from PCCl3 wild type, Neg#1 and 4 D1-65 PCCl3 clones. GAPDH cDNA was amplified for normalization. (B) ^{125}I (1 mCi/ml) uptake in wild type PCCl3 preincubated with or without $NaClO_4$. After washing, the cells were lyzed with 1 N NaOH and the ^{125}I was counted with a gamma Wizard Counter (Perkin-Elmer). The radioactivity was normalized to protein concentration and expressed in percentage of the uptake by wild type PCCl3.

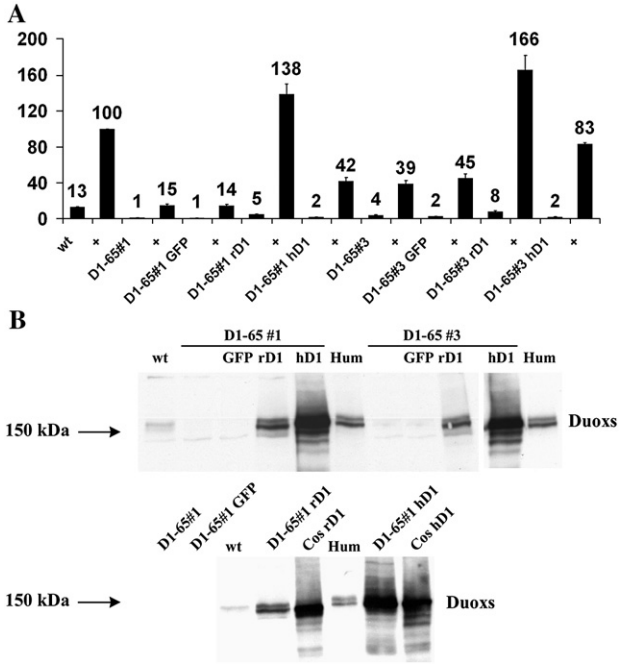


Fig. 8 – Rescue of H_2O_2 generation in D1-65 PCCl3 clones. (A) H_2O_2 generation in rDuox-transduced PCCl3 cells. D1-65#1 and D1-65#3, rat Duox1 knock-down PCCl3 cells, were infected with pWXLd-GFP, pWXLd-rD1 or pWXLd-hD1 lentivirus. H_2O_2 measurements were performed after stimulation (+) or not with 1 μM ionomycin during 2 h and 30 min at 37 °C. All samples were tested in duplicate and the level of H_2O_2 was represented in percentage of the value obtained for ionomycin stimulated wild type PCCl3. Graph is representative of two independent experiments. (B) Duox protein expression in transduced cells. 15 μg of total protein extracts were separated in a 6% SDS–PAGE gel. Duox proteins were immunodetected with the I2 antibody at a dilution of 1/16,000. wt: wild type PCCl3 cells: Hum: 5 μg of total protein extracts of human thyrocyte; Cos rD1 and Cos hD1: 5 μg of total protein extracts from Cos transfected with rat or human Duox1 in pcDNA3.0 by FuGENE6 method.

Cos-7 cells transfected with Duox1 which only expressed the immature high mannose form as previously described (Fig. 8B, lower panel) [14,33].

Discussion

Both Duox1 and Duox2 mRNAs are highly expressed in the thyroid of mammals [12,20,22,25]. The respective role of the two proteins encoded by these two genes in the same cell remains an open question. The lack of selective antibodies has not yet allowed to clarify if these two proteins are actually co-expressed in the thyroid and if both are active. Some arguments suggest that, in human thyroid, Duox2 has a main function in the generation of H_2O_2. Biallelic mutations in *Duox2* gene encoding a truncated protein supposed to be inactive were responsible for permanent and severe congenital hypothyroidism with iodide organification defect [26–28]. Duox1 and Duox2 mRNAs are also transcribed in other tissues like in the airway epithelium where their expression is differentially up-regulated; Duox1 by IL-4 and IL-3 and Duox2 by IFN-γ [16,18,37–39]. In the thyroid, the regulation by the thyroid-stimulating hormone (TSH) is less discriminating: in dog and pig thyroid, TSH simultaneously up-regulates mRNA of Duox1 and Duox2 at the same order of magnitude but this up-regulation is not observed in human thyroid [12,13]. In other tissues, one Duox is mainly expressed: Duox1 in trachea [18] and Duox2 in the salivary gland [18] and in the epithelial cells along the digestive tract [17] but nothing is known about the regulation of their expression or activity.

Silencing specifically Duox1 or Duox2 with siRNAs was a way to distinguish the respective role of the two proteins in the generation of H_2O_2 in the thyroid cells. We chose to silence Duox1 or Duox2 in a differentiated rat thyroid cell line, PCCl3, which possesses a Ca^{2+}-dependent H_2O_2-generating system. PCCl3 cells, like FRTL5 or WRT, are widely used as thyroid models [30,34]. They present properties ascribed to normal differentiated thyrocytes such as TSH dependence for growth and functions of differentiation: iodide uptake, *Tg* and *TPO* gene transcription and H_2O_2 production. However, they also differ in numerous ways: absence of iodide organification, different responses to growth stimuli, and so on [40]. Duox2 cDNA has been identified in FRTL5 (AF237962) [34] as well as Duox1 (AF542180). PCCl3 cells were particularly used in this study for their strong and constant H_2O_2 production in response to Ca^{2+}-stimulated pathway [14]. We observed that siRNAs against Duox1 interfere efficiently and selectively with Duox1 expression at the level of mRNA leading to a decrease of protein expression (supposed to be Duox1) and H_2O_2 production. NIS function, assessed by measuring the transcript level and the iodide uptake, was not affected by the expression of siRNAs against Duox1 meaning that these siRNAs do not interfere with the thyroid function. Surprisingly, siRNAs, which efficiently silence Duox2 in the CHO heterologous system, were unable to inhibit PCCl3 endogenous Duox expression or H_2O_2 production. These results can be explained by the low amount of Duox2 transcript detected in these cells compared to Duox1 and therefore its negligible contribution to H_2O_2 production. The same phenomenon has already been described in rat airway epithelial cells using siRNAs silencing Duox1 which is less abundant than Duox2 in these cells [38].

The high level of Duox1 expression relative to Duox2 expression in PCCl3 was not observed in thyroids of Sprague-Dawley rats. Duox2 was obviously more abundant in the thyroid tissue than in the cell line but it is noteworthy that this ratio was not observed in another study. Indeed Moskwa et al. have observed a higher level of Duox1 compared to Duox2 in thyroid tissue but the authors did not mention the strain of rat used [38]. These results show that the chosen model, PCCl3, is only informative for Duox1 and may not be fully representative for what happens *in vivo*. This shows again that such cell lines should be validated for any variable studied when used as models [40]. Nevertheless the results demonstrate that Duox1 alone is able to generate H_2O_2 and that the amount of Duox1 present in PCCl3 cells is sufficient to generate a calcium-dependent H_2O_2 production.

Transfection of Duox cDNAs in non-thyroid cells does not lead to functional NADPH oxidases as assessed by H_2O_2

generation [14]. During the completion of this work, DuoxA1 and DuoxA2 (Duox activators) have been characterized as the maturation factors of Duox proteins [29]. DuoxA2 resides in the endoplasmic reticulum compartment. It is essential for Duox2 to undergo glycosylations necessary for its maturation through the Golgi and its correct expression at the plasma membrane where it is functional. We have observed that Duox1 co-expressed with DuoxA1 in heterologous systems is functional (unpublished data). This shows that the expression of Duox1 alone with DuoxA1 would be sufficient to explain the dual oxidase activity measured in PCCl3. The functional activity *in vivo* of the couple Duox/DuoxA can be the consequence of the particular genomic organization of the *Duox/DuoxA* genes in a head to head orientation. They most probably share a bidirectional promoter since DuoxA and Duox transcripts are expressed in the same tissues. This has already been described for the genes of the type IV collagen α-chain of the human basement membrane and of the histones H2A/H2B [41,42]. Following this hypothesis, one could expect the DuoxA1/DuoxA2 mRNA ratio would be in favor of DuoxA1 in PCCl3 cells. This seems not to be the case since DuoxA1 and DuoxA2 present rather similar levels of mRNA expression in PCCl3 cells (unpublished data).

The use of lentivirus technology allows to obtain suppression in a high percentage of PCCl3 which are known difficult to be transfected by classical liposome-mediated transfection methods. The re-introduction of Duox1 in PCCl3 cells rDuox1 knocked-down reversed the phenotype of the cells with re-expression of Duox1 correctly N-linked glycosylated and production of H_2O_2 comparable to wild type cells. This indicates again that ectopic expression of Duox1 alone with endogenous DuoxA1 is able to reconstitute a functional H_2O_2-generating system. The lower potency of human Duox1 to increase H_2O_2 generation in PCCl3 compared to rat Duox1 could arise from species specificities in the Duox/DuoxA association complex.

Nevertheless, the main role attributed to Duox1 does not rule out any function of Duox2 *in vivo* since Duox2 mRNA is more expressed than Duox1 mRNA in rat thyroid tissue as well as in human thyroid tissue. The present data suggest that the NADPH oxidase activity can be associated to one Duox/DuoxA couple in the thyroid. Either Duox1 or Duox2 could play the main role depending on the species and the experimental system investigated. In the case of PCCl3 thyroid cells, Duox1 is highly expressed and is responsible for the generation of H_2O_2.

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REFERENCES

[1] G. Dai, O. Levy, N. Carrasco, Cloning and characterization of the thyroid iodide transporter, Nature 379 (1996) 458–460.
[2] S. Eskandari, D.D. Loo, G. Dai, O. Levy, E.M. Wright, N. Carrasco, Thyroid Na^+/I^- symporter. Mechanism, stoichiometry, and specificity, J. Biol. Chem. 272 (1997) 27230–27238.
[3] N. Uyttersprot, N. Pelgrims, N. Carrasco, C. Gervy, C. Maenhaut, J.E. Dumont, F. Miot, Moderate doses of iodide in vivo inhibit cell proliferation and the expression of thyroperoxidase and Na^+/I^- symporter mRNAs in dog thyroid, Mol. Cell. Endocrinol. 131 (1997) 195–203.
[4] J.E. Dumont, C. Maenhaut, D. Christophe, Vassart P.P. Roger, The phylogeny, ontogeny, anatomy and regulation of the iodine metabolizing thyroid, Thyroid Dis. (2005) (www.Thyroidmanager.org).
[5] D.A. Scott, R. Wang, T.M. Kreman, V.C. Sheffield, L.P. Karniski, The Pendred syndrome gene encodes a chloride–iodide transport protein, Nat. Genet. 21 (1999) 440–443.
[6] V.C. Sheffield, Z. Kraiem, J.C. Beck, D. Nishimura, E.M. Stone, M. Salameh, O. Sadeh, B. Glaser, Pendred syndrome maps to chromosome 7q21–34 and is caused by an intrinsic defect in thyroid iodine organification, Nat. Genet. 12 (1996) 424–426.
[7] N.M. Alexander, Purification of bovine thyroid peroxidase, Endocrinology 100 (1977) 1610–1620.
[8] B. Corvilain, J. Van Sande, E. Laurent, J.E. Dumont, The H_2O_2-generating system modulates protein iodination and the activity of the pentose phosphate pathway in dog thyroid, Endocrinology 128 (1991) 779–785.
[9] C. Dupuy, J. Kaniewski, D. Deme, J. Pommier, A. Virion, NADPH-dependent H_2O_2 generation catalyzed by thyroid plasma membranes. Studies with electron scavengers, Eur. J. Biochem. 20;185 (1989) 597–603.
[10] C. Dupuy, A. Virion, R. Ohayon, J. Kaniewski, D. Deme, J. Pommier, Mechanism of hydrogen peroxide formation catalyzed by NADPH oxidase in thyroid plasma membrane, J. Biol. Chem. 266 (1991) 3739–3743.
[11] R. Ameziane-El-Hassani, S. Morand, J.L. Boucher, Y.M. Frapart, D. Apostolou, D. Agnandji, S. Gnidehou, R. Ohayon, M.S. Noel-Hudson, J. Francon, K. Lalaoui, A. Virion, C. Dupuy, Dual oxidase-2 has an intrinsic Ca^{2+}-dependent H_2O_2-generating activity, J. Biol. Chem. 280 (2005) 30046–30054.
[12] X. De Deken, D. Wang, M.C. Many, S. Costagliola, F. Libert, G. Vassart, J.E. Dumont, F. Miot, Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family, J. Biol. Chem. 275 (2000) 23227–23233.
[13] C. Dupuy, R. Ohayon, A. Valent, M.S. Noel-Hudson, D. Deme, A. Virion, Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs, J. Biol. Chem. 274 (1999) 37265–37269.
[14] X. De Deken, D. Wang, J.E. Dumont, F. Miot, Characterization of ThOX proteins as components of the thyroid H(2)O(2)-generating system, Exp. Cell Res. 273 (2002) 187–196.
[15] D. Wang, D. De, X.M. Milenkovic, Y. Song, I. Pirson, J.E. Dumont, F. Miot, Identification of a novel partner of duox: EFP1, a thioredoxin-related protein, J. Biol. Chem. 280 (2005) 3096–3103.
[16] R.W. Harper, C. Xu, J.P. Eiserich, Y. Chen, C.Y. Kao, P. Thai, H. Setiadi, R. Wu, Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th_1 and Th_2 cytokines in respiratory tract epithelium, FEBS Lett. 579 (2005) 4911–4917.
[17] R.A. El Hassani, N. Benfares, B. Caillou, M. Talbot, J.C. Sabourin, V. Belotte, S. Morand, S. Gnidehou, D. Agnandji, R. Ohayon, J. Kaniewski, M.S. Noel-Hudson, J.M. Bidart, M. Schlumberger, A. Virion, C. Dupuy, Dual oxidase2 is

expressed all along the digestive tract, Am. J. Physiol.: Gastrointest. Liver Physiol. 288 (2005) G933–G942.
[18] M. Geiszt, J. Witta, J. Baffi, K. Lekstrom, T.L. Leto, Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense, FASEB J. 17 (2003) 1502–1504.
[19] E.M. Ha, C.T. Oh, Y.S. Bae, W.J. Lee, A direct role for dual oxidase in *Drosophila* gut immunity, Science 310 (2005) 847–850.
[20] J. Pachucki, D. Wang, D. Christophe, F. Miot, Structural and functional characterization of the two human *ThOX/Duox* genes and their 5′-flanking regions, Mol. Cell. Endocrinol. 214 (2004) 53–62.
[21] J.C. Moreno, E. Pauws, A.H. van Kampen, M. Jedlickova, J.J. de Vijlder, C. Ris-Stalpers, Cloning of tissue-specific genes using serial analysis of gene expression and a novel computational substraction approach, Genomics 75 (2001) 70–76.
[22] B. Caillou, C. Dupuy, L. Lacroix, M. Nocera, M. Talbot, R. Ohayon, D. Deme, J.M. Bidart, M. Schlumberger, A. Virion, Expression of reduced nicotinamide adenine dinucleotide phosphate oxidase (ThoX, LNOX, Duox) genes and proteins in human thyroid tissues, J. Clin. Endocrinol. Metab. 86 (2001) 3351–3358.
[23] A.C. Gerard, M.C. Many, C. Daumerie, S. Costagliola, F. Miot, J.J. DeVijlder, I.M. Colin, J.F. Denef, Structural changes in the angiofollicular units between active and hypofunctioning follicles align with differences in the epithelial expression of newly discovered proteins involved in iodine transport and organification, J. Clin. Endocrinol. Metab. 87 (2002) 1291–1299.
[24] A.C. Gerard, C. Daumerie, C. Mestdagh, S. Gohy, C. De Burbure, S. Costagliola, F. Miot, M.C. Nollevaux, J.F. Denef, J. Rahier, B. Franc, J.J. De Vijlder, I.M. Colin, M.C. Many, Correlation between the loss of thyroglobulin iodination and the expression of thyroid-specific proteins involved in iodine metabolism in thyroid carcinomas, J. Clin. Endocrinol. Metab. 88 (2003) 4977–4983.
[25] L. Lacroix, M. Nocera, C. Mian, B. Caillou, A. Virion, C. Dupuy, S. Filetti, J.M. Bidart, M. Schlumberger, Expression of nicotinamide adenine dinucleotide phosphate oxidase flavoprotein *DUOX* genes and proteins in human papillary and follicular thyroid carcinomas, Thyroid 11 (2001) 1017–1023.
[26] J.C. Moreno, H. Bikker, M.J. Kempers, A.S. van Trotsenburg, F. Baas, J.J. de Vijlder, T. Vulsma, C. Ris-Stalpers, Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism, N. Engl. J. Med. 347 (2002) 95–102.
[27] V. Varela, C.M. Rivolta, S.A. Esperante, L. Gruneiro-Papendieck, A. Chiesa, H.M. Targovnik, Three mutations (p.Q36H, p.G418fsX482, and g.IVS19-2A>C) in the dual oxidase 2 gene responsible for congenital goiter and iodide organification defect, Clin. Chem. 52 (2006) 182–191.
[28] M.C. Vigone, L. Fugazzola, I. Zamproni, A. Passoni, S. Di Candia, G. Chiumello, L. Persani, G. Weber, Persistent mild hypothyroidism associated with novel sequence variants of the *DUOX2* gene in two siblings, Human Mutat. 26 (2005) 395.
[29] H. Grasberger, S. Refetoff, Identification of the maturation factor for dual oxidase. Evolution of an eukaryotic operon equivalent, J. Biol. Chem. 281 (2006) 18269–18272.
[30] A. Fusco, M.T. Berlingieri, P.P. Di Fiore, G. Portella, M. Grieco, G. Vecchio, One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes, Mol. Cell. Biol. 7 (1987) 3365–3370.
[31] B. Benard, J. Brault, Production of peroxide in the thyroid, Union Med. Can. 100 (1971) 701–705.
[32] L.S. Minamide, J.R. Bamburg, A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents, Anal. Biochem. 190 (1990) 66–70.
[33] S. Morand, M. Chaaraoui, J. Kaniewski, D. Deme, R. Ohayon, M.S. Noel-Hudson, A. Virion, C. Dupuy, Effect of iodide on nicotinamide adenine dinucleotide phosphate oxidase activity and Duox2 protein expression in isolated porcine thyroid follicles, Endocrinology 144 (2003) 1241–1248.
[34] C. Dupuy, M. Pomerance, R. Ohayon, M.S. Noel-Hudson, D. Deme, M. Chaaraoui, J. Francon, A. Virion, Thyroid oxidase (*THOX2*) gene expression in the rat thyroid cell line FRTL-5, Biochem. Biophys. Res. Commun. 277 (2000) 287–292.
[35] T. Dull, R. Zufferey, M. Kelly, R.J. Mandel, M. Nguyen, D. Trono, L. Naldini, A third-generation lentivirus vector with a conditional packaging system, J. Virol. 72 (1998) 8463–8471.
[36] R. Zufferey, T. Dull, R.J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, D. Trono, Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery, J. Virol. 72 (1998) 9873–9880.
[37] R. Forteza, M. Salathe, F. Miot, R. Forteza, G.E. Conner, Regulated hydrogen peroxide production by Duox in human airway epithelial cells, Am. J. Respir. Cell Mol. Biol. 32 (2005) 462–469.
[38] P. Moskwa, D. Lorentzen, K.J. Excoffon, J. Zabner, P.B. McCray Jr., W.M. Nauseef, C. Dupuy, B. Banfi, A novel host defense system of airways is defective in cystic fibrosis, Am. J. Respir. Crit Care Med. 175 (2007) 174–183.
[39] U.V. Wesley, P.F. Bove, M. Hristova, S. McCarthy, V.d. van, Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1, J. Biol. Chem. (2006).
[40] T. Kimura, A. Van Keymeulen, J. Golstein, A. Fusco, J.E. Dumont, P.P. Roger, Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models, Endocr. Rev. 22 (2001) 631–656.
[41] R.A. Sturm, S. Dalton, J.R. Wells, Conservation of histone H2A/H2B intergene regions: a role for the H2B specific element in divergent transcription, Nucleic Acids Res. 16 (1988) 8571–8586.
[42] J. Zhou, M. Ding, Z. Zhao, S.T. Reeders, Complete primary structure of the sixth chain of human basement membrane collagen, alpha 6(IV). Isolation of the cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains, J. Biol. Chem. 269 (1994) 13193–13199.