Intercellular HOCl-mediated Apoptosis Induction in Malignant Cells: Interplay Between NOX1-Dependent Superoxide Anion Generation and DUOX-related HOCl-generating Peroxidase Activity

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Abstract. *Intercellular apoptosis-inducing HOCl signaling is discussed as a control step during oncogenesis. It is defined as a sophisticated interplay between transformed target cells and non-transformed or transformed effector cells. In this study, transformed target cells were seeded as clumps of high local cell density, but low total cell number. They were surrounded by large numbers of effector cells, seeded at low local density. This spatially defined experimental arrangement allowed study of the impact of siRNA-mediated knockdown of NADPH oxidase 1 (NOX1) or dual oxidase 1 (DUOX1) on intercellular HOCl signaling. Our data show that the target function of transformed cells is defined as expression of NOX1 and subsequent extracellular superoxide anion generation. The NOX domain of DUOX1 does not contribute to the target function. The peroxidase domain of DUOX1 is released from transforming growth factor β1-treated non-transformed and transformed cells and acts in trans as HOCl-synthesizing peroxidase. These findings clarify the biochemical source of HOCl during HOCl-mediated signaling.*

A relevant biological function of HOCl was first shown for the anti-bacterial action of professional phagocytes such as neutrophils (1-12). In these cells, the interplay between NADPH oxidase2 (NOX2) in the phagosomal membrane and myeloperoxidase (MPO) allows the synthesis of HOCl (i) $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$; ii) $MPOFe^{III} + H_2O_2 \rightarrow MPOFe^{IV}{=}O^{\bullet+} + H_2O$; iii) $MPOFe^{IV}{=}O^{\bullet+} + Cl^- + H^+ \rightarrow MPOFe^{III} + HOCl$) (3, 4, 6). The subsequent interaction of HOCl with superoxide anions results in site-specific generation of hydroxyl radicals ($HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + Cl^- + O_2$), that contribute to the action of neutrophils against pathogens (5, 13-15).

A distinct antitumor cell effect of HOCl generated by the peroxidase (POD)/halide system is well-established (8-12). It may be one of the central mechanisms underlying the antitumor effect of neutrophils *in vivo* (16). The mechanism of HOCl-mediated antitumor action, and especially the basis of its selectivity for malignant cells, remained enigmatic until extracellular superoxide anion production through oncogene-controlled membrane-associated NOX1 was discovered as a hallmark of the transformed state of cells (17-25). It was then shown that the reaction between HOCl and extracellular superoxide anions that are generated specifically by malignant cells causes site-specific formation of apoptosis-inducing hydroxyl radicals ($HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + Cl^- + O_2$) (23, 24, 26-28). Therefore, exogenous HOCl (in the micromolar concentration range) induces apoptosis in transformed cells and tumor cells, whereas it does not affect non-transformed cells, as these lack extracellular superoxide anion production.

Under suitable conditions, a co-culture of transformed and non-transformed cells or a population of transformed cells alone, exhibit HOCl synthesis and subsequent apoptosis induction selectively in transformed cells through HOCl-dependent reactions. This intercellular signaling pathway has been termed the HOCl signaling pathway (23, 27, 29). It is based on extracellular superoxide anion generation by the transformed target cells and on the release of a *trans*-acting POD supplied by non-transformed or transformed effector cells. Superoxide anions from transformed cells are the basis for the generation of hydrogen peroxide, which is then used as a substrate by the POD. The resultant HOCl reacts with

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transformed cell-derived superoxide anions, yielding apoptosis-inducing hydroxyl radicals in close vicinity to the membrane of the transformed target cells. This site-specific generation of hydroxyl radicals is the basis for selectivity of apoptosis induction with respect to the malignant phenotype of cells.

Tumor cells prevent HOCl synthesis through decomposition of hydrogen peroxide by membrane-associated catalase and therefore are protected against the HOCl signaling pathway, unless their membrane-associated catalase is inhibited (23, 25). However, tumor cells respond to exogenously added HOCl with apoptosis induction as they generate superoxide anions that react with HOCl and form hydroxyl radicals (28). The identity of the HOCl-synthesizing POD that drives the HOCl signaling pathway in many cell systems studied by our group remained enigmatic so far. However, due to its action in *trans*, it was obvious that the responsible HOCl-synthesizing POD must be released from nontransformed, transformed and tumor cells, as these have established effector function in trans. In reconstitution experiments, the function of the HOCl-synthesizing POD was fully mimicked by MPO (26). However, as MPO expression is essentially restricted to neutrophils and monocytes, this enzyme was not a reasonable candidate to explain the POD activity involved in HOCl signaling that we detected in cellular systems such as fibroblasts, epithelial cells, neuronal cells and others (23, 25, 27, Bauer, unpublished results). One attractive potential candidate was dual oxidase (DUOX) (30-34) as i) it has an aminoterminal POD ectodomain in addition to a NOX domain, ii) the activity of the DUOX POD domain is inhibited by the mechanism-based POD inhibitor 4-aminobenzoyl hydrazide (ABH) (30), and iii) the comparison of the sequence of the POD domain of DUOX with MPO showed a strong structural relationship between the two enzymes (30). Here we present data that demonstrate the interplay between NOX1 of malignant target cells and DUOX or a DUOX-related POD from nontransformed or transformed effector during intercellular apoptosis-inducing reactive oxygen species (ROS) signaling, a potential control step during oncogenesis.

Materials and Methods

Materials. The catalase inhibitior 3-aminotriazole (3-AT), catalase from bovine liver, NaOCl (for the generation of HOCl), the broad spectrum matrix metalloproteinase inhibitor (R)-*N*4-hydroxy-*N*1-[(S)-2-(1*H*-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide (Galardin; GM6001) (35), isopropyl-β-D-thiogalactopyranosid (IPTG), the hydroxyl radical scavenger mannitol, manganese-containing superoxide dismutase (Mn-SOD) from *Escherichia coli*, the inhibitor of NO synthase (NOS) N-omega-nitro-L-arginine methylester hydrochloride (L-NAME), the HOCl scavenger taurine, the NO donor sodium nitroprusside (SNP), hydrogen peroxide-generating glucose oxidase (GOX) from *Aspergillus niger*, and MPO from human leucocytes) were obtained from Sigma Aldrich (Schnelldorf, Germany).

Transforming growth factor β1 (TGFβ1) was purified from human platelets (36) and kept as a stock solution of 1.5 μg/ml in Eagle's Minimum Essential Medium (EMEM) plus 5% fetal bovine serum (FBS) at −20˚C.

The catalase mimetic EUK-8 [manganese *N,N'*-bis(salicylidene)ethylenediamine chloride] was obtained from Cayman chemicals, Ann Arbor, Michigan, U.S.A. through Merck Biosciences GmbH, Schwalbach/Ts, Germany. The peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) chloride (FeTPPS) was obtained from Calbiochem/Merck Biosciences GmbH, Schwalbach/Ts, Germany. The mechanism-based POD inhibitor ABH was obtained from Acros Organics, Geel, Belgium.

Media for cell culture. Cells were either kept in Eagle's minimal essential medium (EMEM), containing 5% FBS (Biochrom, Berlin, Germany) or in RPMI-1640 medium, containing 10% FBS, as indicated for the respective cell lines. FBS was heated for 30 minutes at 56˚C prior to use. Both media were supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). All supplements were obtained from Biochrom, Berlin, Germany. Cell culture was performed in plastic tissue culture flasks. Cells were passaged once or twice weekly.

Cells. Nontransformed rat fibroblasts 208F (37), their derivative transformed through constitutive expression of v-src (208Fsrc3) (38), 208F cells with an inducible Harvey rat sarcoma (*HRAS*)-oncogene (IR-1) (21, 39) and nontransformed rat ovary surface epithelial cells (ROSE 199) were a generous gift from Dr C. Sers and Dr R. Schäfer, Berlin, Germany.

For the establishment of IR-1 cells, immortalized 208F rat fibroblasts were transfected with the *HRAS* oncogene under the control of the *SV40* promoter and the lac operator sequence, the *lac* repressor gene and p*SV2neo* (39). For induction of RAS expression, the cells received 10 mM IPTG 48 h before, as well as during the experiment. Throughout this article, IR-1 cells with an induced *HRAS* oncogene are termed IR-1 RAS. IR-1 cells with and without induction of the RAS oncogene had been previously used in studies for intercellular ROS-mediated apoptosis induction (21).

208F cells, their transformed derivatives and ROSE 199 cells were cultured in EMEM, with 5% FBS and supplemented as indicated above.

The gastric carcinoma cell line MKN-45 was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cells were grown in suspension, with some cells attaching to the plastic culture dish, in RPMI-1640, with 10% serum and supplements as described above. Care was taken to avoid cell densities below 300,000/ml and above 10^6/ml. Under optimal conditions, the percentage of spontaneous apoptosis induction was less than 1%.

SiRNA-mediated knockdown of NOX1 and DUOX1. Control siRNA (siCo), siRNA against human NOX1 (siNOX1) and human *DUOX1* (siDUOX1) were obtained from Qiagen (Hilden, Germany) with the following sequences: siCo: sense: r(UUCUCCGAACGUGUC ACGU)dTdT, antisense: ACGUGACACGUUCGGAGAA)dTdT; siNOX1: sense: r(GACAAAUACUACUACACAA)dTdT, antisense: r(UUGUGUAGUAGUAUUUGUC)dGdG; and siDUOX1: sense: r(AGU CUA ACA CCA CAA CUA A)dTdT, antisense: r(UUA GUU GUG GUG UUA GAC U)dGdG.

siRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 μM. Suspensions were heated at 90˚C for 1 minute, followed by incubation at 37˚C for 60 min. Aliquots were stored at −20˚C.

Before transfection, 88 μl of medium without serum and without antibiotics were mixed with 12 μl Hyperfect solution (Qiagen) and 1.2 μl specific siRNA or control siRNA. The mixture was treated by a Vortex mixer for a few seconds and then allowed to sit for 10 minutes. It was then gently and slowly added to 300,000 MKN-45 cells in 1 ml RPMI-1640 medium containing 10% FBS and antibiotics (12-well plates) or to 2×10^5 208F or 208Fsrc3 cells/well (6-well plates) plus 2.3 ml medium supplemented with 5% FBS and antibiotics. Thus the concentration of siRNA was 24 nM for MKN-45 cells in suspension and 10 nM for 208F and 208Fsrc3 cells in monolayer. The cells were incubated at 37˚C in 5% CO_2 for 24 hours. Transfected cells were centrifuged and resuspended in fresh medium at the required density before use.

Determination of the efficiency of siRNA-mediated knockdown. The siRNA transfection system as described above had been optimized to allow a reproducible transfection efficiency of more than 95% of the cells and to avoid toxic effects (Bauer, unpublished result). We used a functional quantitation of knockdown efficiency, in line with the requirements of our experimental approach.

Functional knockdown of *NOX1* by siNOX1 was determined in 208Fsrc3 and MKN-45 cells through direct quantitation of superoxide anion production by siCo and siNOX1-transfected cells 24 h after transfection, following the protocol described byTemme and Bauer (40). Functional knockdown was at least 94%. The same result was obtained with a second siRNA directed towards *NOX1* (data not shown).

Functional knockdown of *DUOX1* by siDUOX1 was determined for 208Fsrc3 cells by direct quantitation of POD release from siCo and siDUOX1-transfected cells 24 h after transfection, using a recently established competition test for the determination of released POD (41) which is described below. SiDUOX-mediated functional knockdown of DUOX1 was 88%. This result was confirmed through transfection with a second siRNA directed towards DUOX1 (data not shown).

Determination of the percentage of apoptotic cells. The precise quantitative determination of the percentage of apoptotic cells in duplicate assays was the read-out for experiments that quantified i) autocrine apoptotic selfdestruction (Figure 1A-F; Figures 7, 8, ii) apoptosis induction by addition of exogenous HOCl (Figure 1G), iii) apoptosis induction in clumps of target cells surrounded by disperse effector cells (Figures 3-6) and iv) the release of POD in a competition assay (Figure 9).

The percentage of apoptotic cells was determined by inverted phase-contrast microscopy based on the classical criteria for apoptosis, *i.e*. nuclear condensation or fragmentation and membrane blebbing (25, 42, 43). At least 2×200 cells were scored for each point of measurement in duplicate assays. Comparative analysis with several cell lines had shown that nuclear condensation/fragmentation as determined by inverse phase-contrast microscopy were correlated to intense staining with bisbenzimide, and to DNA strand breaks, detectable by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction (44-48). However, the TUNEL reaction was not used for routine quantitation, as the distinct steps during preparation of the samples caused a marked loss specifically of apoptotic cells.

Direct measurement of autocrine apoptotic self-destruction. Cells to be tested were seeded at a density of 12,500 cells/well (208F, 208Fsrc3, MKN-45 cells) or 6,000 cells/well (IR-1 cells or IR1-RAS) in 96-well tissue culture clusters and were overlaid with 100 μl of complete medium. 208F, 208Fsrc3 and IR-1 cells with and without RAS induction received 20 ng/ml TGFβ1 in addition. MKN-45 cells received either no 3-AT, 75 mM 3-AT, or 3-AT at 6.2-150 mM. The following inhibitors of HOCl signaling were applied as indicated: 60 U/ml of MnSOD, 50 mM of the HOCl scavenger taurine, 150 μM of the mechanism-based POD inhibitor ABH or 10 mM of the hydroxyl radical scavenger mannitol. In all experiments, assays were performed in duplicate. The percentage of apoptotic cells were determined after 4 h (MKN-45 cells), 24 h (208F and 208Fsrc3 cells) or 41 h (IR-1 cells). Modifications of the standard procedure are indicated in the respective figure legends.

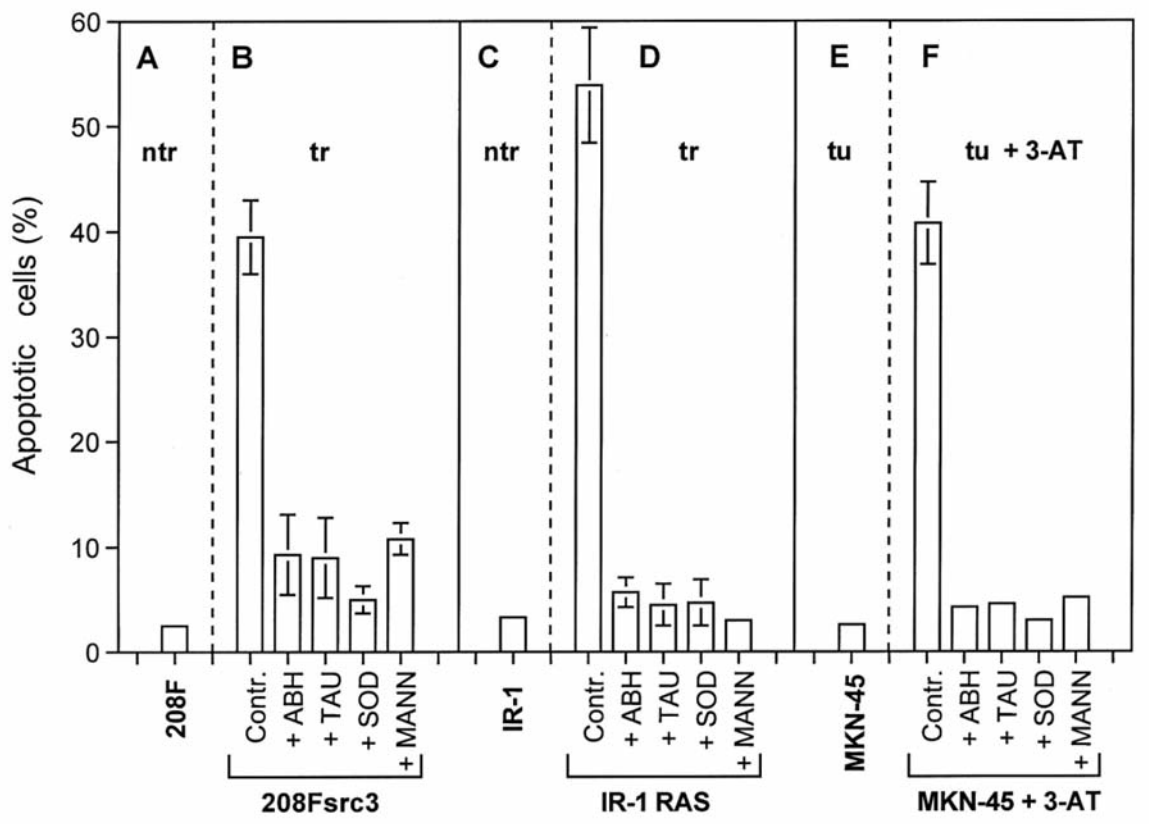
Apoptosis induction by addition of exogenous HOCl. A total of 5×10^3 transformed 208Fsrc3 or non-transformed 208F cells/100 μl complete medium, or 12,500 MKN-45 gastric carcinoma cells/100 μl complete medium remained untreated or received 430 μM HOCl (208F and 208Fsrc3 cells) or 125 μM HOCl (MKN-45 cells). To determine the role of superoxide anions for HOCl-mediated apoptosis induction, 100 U/ml MnSOD were added prior to HOCl addition to not. After 2 h, the percentages of apoptotic cells in duplicate assays were determined as described above.

Co-culture of clumps of target cells surrounded by disperse effector cells. The principle of this assay is based the interaction between transformed target cells that are seeded as clumps of low cell number, but high local cell density, and surrounding nontransformed or transformed effector cells of high cell number, but low local cell density. The high local density of target cells is required for efficient hydrogen peroxide formation through dismutation of target cell-derived superoxide anions, whereas their low cell number causes dilution of POD beyond an effective level. Dispersely seeded effector cells supply target cells with the necessary concentration of POD for the establishment of HOCl signaling, but even if they are transformed, they are not affected by POD due to their low cell density that prevents sufficient formation of hydrogen peroxide. This assay can be used for the measurement of HOCl signaling-dependent intercellular induction of apoptosis in the target cells and is illustrated in Figure 2.

When combined with siRNA-mediated knockdown of specific enzymes such as NOX1 and DUOX1 either in target or effector cells, or with the addition of specific inhibitors of ROS signaling, this spacially and functionally structured experimental system can be used for the detailed dissection of the HOCl signaling pathway, as shown here.

Target cells were seeded as clumps (2,000 cells in 5 μl medium, corresponding to 2,000 cells/8 mm^2) either in 24-well (one clump per well) or in 12-well tissue culture clusters (two clumps per well). After the cells were attached, the clumps were overlaid with 0.5 ml of complete medium and 8,000 effector cells (24-well assays) or 1 ml of complete medium 1 ml of medium and 12,000 effector cells (12-well assays). 208F and 208Fsrc3 cells were used as effector cells in this study. As essential control, clumps of target cells were cultivated in medium in the absence of effector cells. As further control, dispersely seeded effector cells were cultivated in the absence of target cells.

In the experiment shown in Figure 3A, clumps of transformed 208Fsrc3 or nontransformed 208F cells in 0.5 ml medium received either no further addition, 20 ng/ml TGFβ1, 200 mU/ml MPO, 8×10^3



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Cell line	Treatment		
	Control	+ HOCl	+ HOCl + SOD
208F	2.0 ± 0.2	3.2 ± 0.5	2.5 ± 0.5
208Fsrc3	3.6 ± 2	32 ± 2	4 ± 1.2
MKN-45	4.8 ± 0.6	35 ± 0.4	4 ± 0.7

Figure 1. *Autocrine apoptosis induction through HOCl signaling in cells from different stages of multi-step oncogenesis (A-F). Autocrine apoptosis was determined in nontransformed (ntr) 208F cells (A), transformed (tr) 208Fsrc3 cells (B), nontransformed IR-1 cells (C), IR-1 cells transformed through induction of their RAS oncogene (IR1-RAS) (D), MKN-45 tumor cells (tu) (E) and MKN-45 tumor cells in the presence of the catalase inhibitor 3-aminotriazole (3-AT) (tu 3-AT) (F). Autocrine apoptosis induction was only seen in transformed cells (B, D), and in tumor cells in the presence of 3-AT. Autocrine apoptosis induction was inhibited by the peroxidase inhibitor aminobenzoyl hydrazide (ABH), the HOCl scavenger taurine (TAU), superoxide anion-scavenging superoxide dismutase (SOD) and the hydroxyl radical scavenger mannitol (MANN), pointing to the role of HOCl signaling. Apoptosis induction by HOCl (G). Exogenous HOCl induced apoptosis in transformed 208Fsrc3 cells and MKN-45 tumor cells, but not in nontransformed 208F cells. Apoptosis induction by HOCl was dependent on superoxide anions, as it was inhibited by SOD.*

dispersely seeded 208F or 208Fsrc3 cells (in the absence or presence of 20 ng/ml TGFβ1). In addition, clumps of 1000 cells/8 mm2 received 0.5 mM sodium nitroprusside (SNP). The percentage of apoptotic cells was determined after 48 h in duplicate assays. In the experiment shown in Figure 3B, clumps of transformed 208Fsrc3 cells in 0.5 ml medium plus 20 ng/ml TGFβ1 remained without further addition or received 200 mU/ml MPO, 0.5 mU/ml glucose oxidase (GOX), 8,000 dispersely seeded 208Fsrc3 effector cells without or with 150 μM ABH. After 48 h the percentage of apoptotic cells in clumps was determined in duplicate assays. In the control experiment shown in Figure 3C, 8×10^3 208Fsrc3 cells were seeded in 0.5 ml medium in 24 well tissue culture clusters and received either 200 mU/ml MPO, 0.5 mU/ml GOX or no addition. The percentage of apoptotic cells was determined in duplicate assays after 48 h.

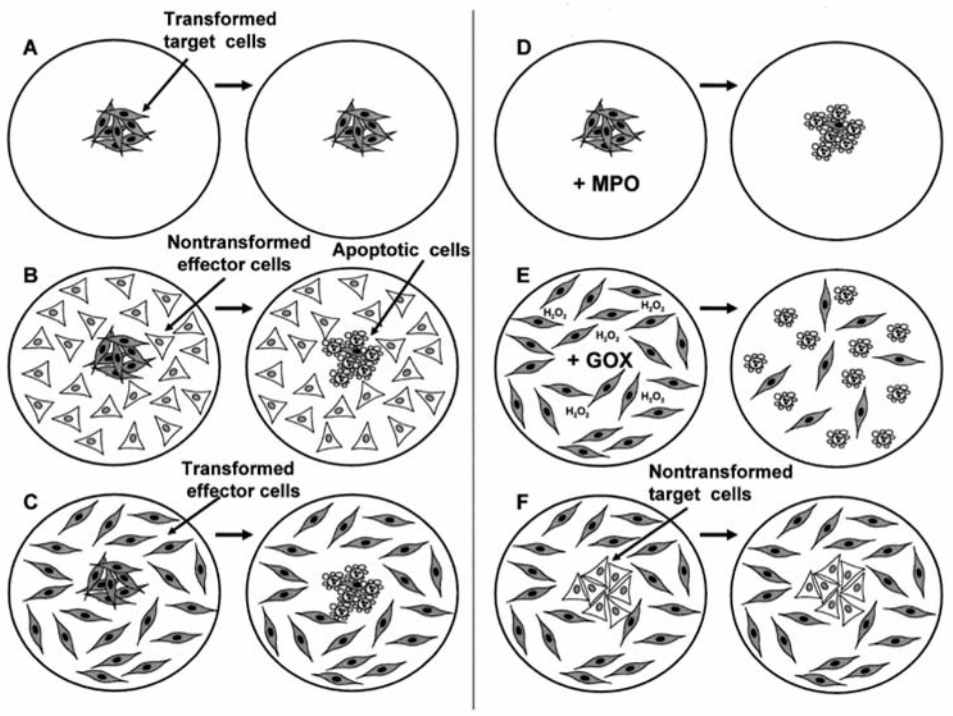

Figure 2. *Schematic presentation of the cell system used for the differentiation between target and effector cell function during intercellular apoptosis induction through HOCl signaling. Transformed target cells were seeded as clumps of high local density, but low total cell number (A). Clumps of transformed cells cultivated alone did not show a significant apoptosis induction, but showed apoptosis induction when they were surrounded by a large number of effector cells (of low local density) (B, C). Effector cells were either nontransformed or transformed. They did not show apoptosis induction within the time of the experiment. Apoptosis induction in transformed target cells was enhanced by the addition of exogenous transforming growth factor- β1 (TGFβ1) (not shown). When nontransformed cells were used as target cells (F), no apoptosis induction was observed. Addition of myeloperoxidase (MPO) to transformed target cells had the same effect as the co-culture with effector cells (D). Dispersely seeded transformed effector cells showed apoptosis induction, when low concentrations of H_2O_2-generating glucose oxidase (GOX) were added (E).*

In the experiment shown in Figure 4, transformed 208Fsrc3 cells, 24 h after transfection with siCo (208Fsrc3siCo), siNOX1 (208Fsrc3siNOX1) or siDUOX1 (208Fsrc3siDUOX1) and nontransformed 208F cells 24 h after transfection with siCo (208FsiCo) were seeded as clumps. Assays (1 ml of complete medium) remained free of additions, or received 20 ng/ml TGFβ1, 12,000 dispersely seeded 208FsiCo cells without further addition or in combination with TGFβ1 and ABH. Other assays received no addition, 20 ng/ml TGFβ1 or 12,000 dispersely seeded 208Fsrc3siCo cells, with TGFβ1 and ABH. After 22 h, the percentage of apoptotic cells was determined in the clumps in duplicate experiments.

In the experiment shown in Figure 5, transformed 208Fsrc3siCo, 208Fsrc3siNOX1 or 208Fsrc3siDUOX1 cells, 24 h after transfection, were seeded as clumps. Assays in 1 ml of complete medium received either 20 ng/ml TGFβ1 alone or TGFβ1 in combination with 12,000 dispersely seeded 208FsiCo, 208FsiNOX1 or 208FsiDUOX1 cells , or 208Fsrc3siCo, 208Fsrc3siNOX1 or 208FsiDUOX1 cells. The percentage of apoptotic cells in clumps was determined after 22 h in duplicate assays.

In the experiment shown in Figure 6, clumps of transformed 208Fsrc3siCo, 208Fsrc3siDUOX1 or 208Fsrc3siNOX1 cells (24 h after transfection) received 1 ml of complete medium plus 20 ng/ml TGFβ1. Assays shown in Figure 6A did not receive effector cells, whereas those shown in Figure 6B-D received 12×10^3 208FsiCo cells. Assays remained without further addition or received 100 U/ml MnSOD, 30 U/ml catalase, 150 μM ABH, 50 mM taurine, 10 mM mannitol or 2.4 mM L-NAME. The percentage of apoptotic cells was determined after 22 h in duplicate assays.

Determination of released POD in a competition assay. This assay depends on HOCl synthesis by relatively high concentrations (75 μM) of the salen-manganese complex EUK-8, utilizing 208Fsrc3 cell-derived hydrogen peroxide (EUK-8 $Mn^{III} + H_2O_2 \rightarrow$ EUK-8 $Mn^{V}O_2^- + H_2O$; EUK-8 $Mn^{V}O_2^- + Cl^- + H+ \rightarrow$ EUK-8 $Mn^{III} + HOCl$) (41, 49; Ophoven and Bauer, unpublished results). The interaction between HOCl and 208Fsrc3 cell-derived superoxide anions results in hydroxyl radical-mediated apoptosis induction in these cells ($HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + O_2 + Cl^-$). As EUK-8 has a very high affinity for hydrogen peroxide which is present in a limited concentration, addition of natural PODs such as MPO or the POD domain of DUOX1 (which have a lower affinity for hydrogen peroxide than EUK-8) (Ophoven and Bauer, unpublished result) does not lead to HOCl synthesis by these PODs, as EUK-8 competes for the required substrate hydrogen peroxide. Therefore, the PODs are confronted with HOCl generated by EUK-8. This triggers the reverse reaction, *i.e.* the decomposition of HOCl ($HOCl + PODFe^{III} \rightarrow Cl^- + H^+ +$

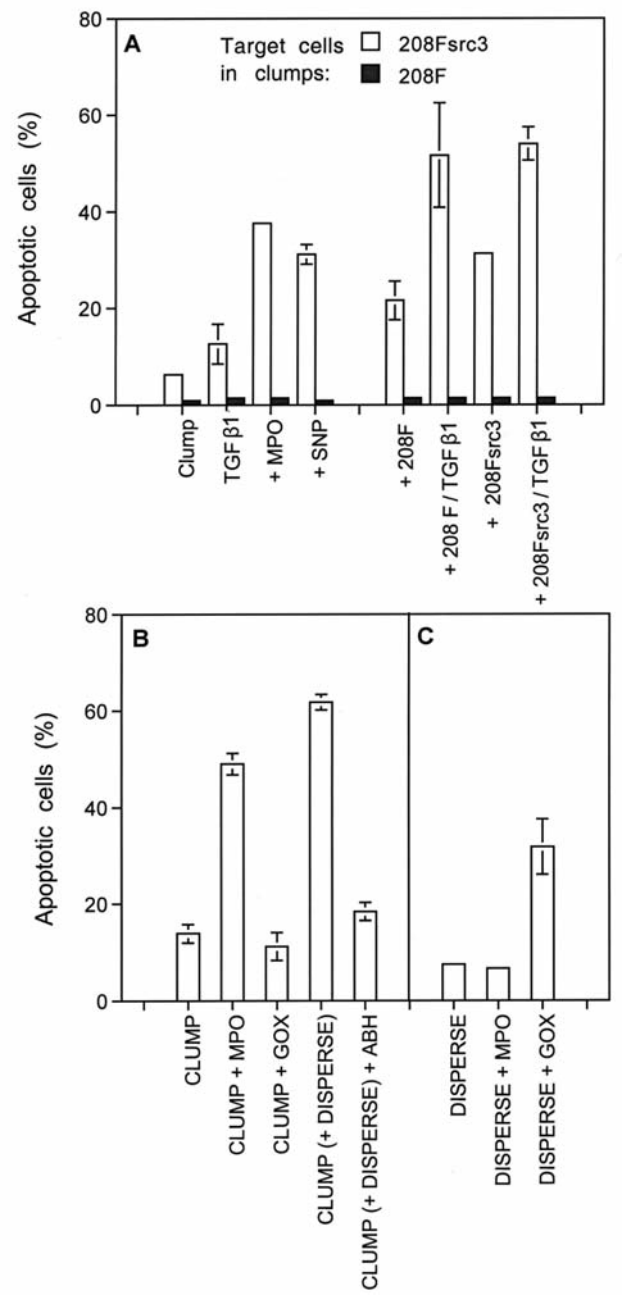

Figure 3. *Specificity and basic requirements for induction of intercellular ROS-mediated apoptosis. A: Apoptosis was induced in clumps of transformed 208Fsrc3 cells in the presence of myeloperoxidase (MPO), the NO donor sodium nitroprusside (SNP) and by surrounding 208F or 208Fsrc3 effector cells. Transforming growth factor-β1 (TGFβ1) enhanced the effect of 208F and 208Fsrc3 cells. Clumps of non-transformed 208F cells did not show apoptosis induction under any condition applied. B. In the presence of TGFβ1, clumps of transformed 208Fsrc3 cells showed apoptosis induction in the presence of MPO or surrounding disperse 208Fsrc3 cells, but not in the presence of glucose oxidase (GOX) or dispersed 208Fsrc3 cells plus peroxidase inhibitor aminobenzoyl hydrazide (ABH). C: Dispersed 208Fsrc3 cells showed no apoptosis induction in the presence of MPO, but did in the presence of glucose oxidase (GOX).*

$PODFe^{IV}=O^{\bullet+}$; $PODFe^{IV}=O^{\bullet+} + O_2^{\bullet-} \rightarrow PODFe^{IV}=O + O_2$; $PODFe^{IV}=O + O_2^{\bullet-} + 2\ H+ \rightarrow PODFe^{III} + H_2O + O_2$). Therefore, increasing concentrations of PODs with lower affinity for hydrogen peroxide than EUK-8 cause a decrease in EUK-8-mediated, HOCl-dependent apoptosis induction in a competitive manner. 5- 10 mU/ml MPO caused approximately 5% competition. This curve of the competition allows quantitation of POD activity, provided the specificity of the competition reactionis ensured by abrogation of competition by the mechanism-based POD inhibitor ABH.

A total of 20,000 208F or 208Fsrc3 cells , 208Fsrc3 cells 24 h after transfection with siCo, siDUOX1 or siNOX1, IR-1 or IR1-RAScells, or ROSE-199 cells were seeded into 24-well tissue culture clusters, 0.5 ml complete medium, without or with 20 ng/ml TGFβ1. Parallel cultures of these cells plus TGFβ1 also received 10 μM galardin. After 24 h at 37˚C, supernatants were collected, centrifuged and stored at −20˚C prior to testing.

Increasing volumes of supernatants were added to 12,500 208Fsrc3 cells in 100 μl complete medium, 96-well tissue culture cluster, containing 75 μM EUK-8 and 20 ng/ml TGFβ1. All assays were performed in duplicate. In parallel, all assays were performed in the presence of ABH. After 7 h, the percentage of apoptotic cells were determined as described above. Apoptosis induction in controls without EUK-8 was below 7% (data not shown), whereas apoptosis induction by EUK-8 in the absence of competing supernatants was between 40-70%.

All ABH-containing assays gave the same result, *i.e.* complete abrogation of inhibition of apoptosis. Inhibition of EUK-8-mediated apoptosis induction by supernatant is indicative of competing POD activity.

Statistical analysis. In all experiments, assays were performed in duplicate. The empirical standard deviation was calculated and is shown in the figures. Absence of standard deviation bars for certain points indicates that the standard deviation was too small to be reported by the graphic program, *i.e.* that results obtained in parallel were nearly identical. Empirical standard deviations were calculated merely to demonstrate how close the results obtained in parallel assays were within the same experiment and not with the intention of statistical analysis of variance, which would require larger numbers of parallel assays. Standard deviations were not calculated between different experiments, due to the usual variation in kinetics of complex biological systems in vitro. The key experiments were repeated more than five times.

The Yates continuity Corrected chi-square test was used for the statistical determination of significances of differences between data.

Results

Autocrine apoptosis induction in cells from different stages of multistep oncogenesis. Cells from defined stages of oncogenesis were tested for autocrine ROS-mediated apoptosis induction. Src oncogene-transformed 208Fsrc3 and IR-1 RAS showed substantial autocrine apoptosis induction ($p<0.001$), but their non-transformed counterparts (parental murine 208F fibroblasts and IR-1 cells without induction of their RAS oncogene) did not (Figure 1A-D). MKN-45 gastric carcinoma cells did not exhibit apoptosis induction unless their recently characterized membrane-associated protective catalase was

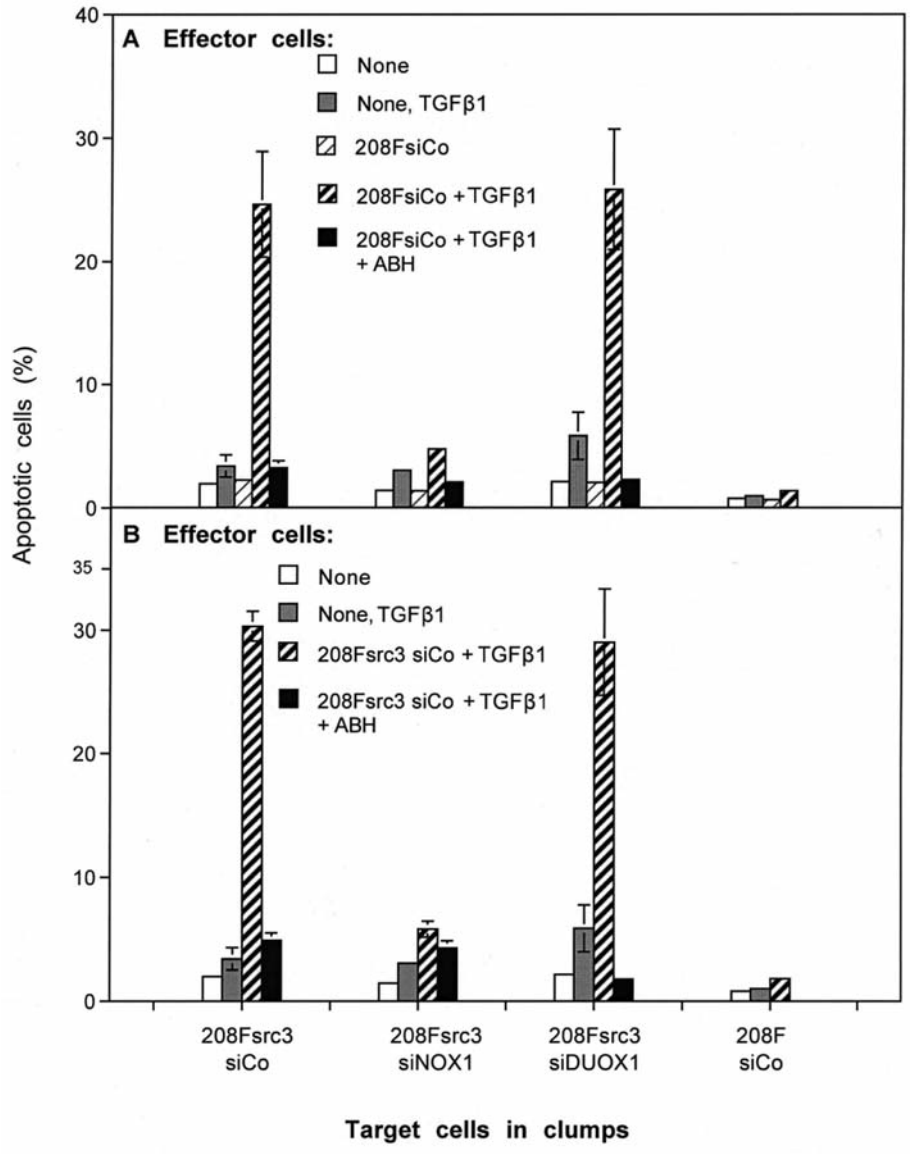

Figure 4. *NADPH oxidase 1 (NOX1) of transformed cells determines target cell specificity, whereas the effector function is independent of the state of transformation. In the presence of transforming growth factor-β1 (TGFβ1), nontransformed 208F and transformed 208Fsrc3 effector cells caused apoptosis induction in transformed 208Fsrc3 target cells transfected with control siRNA (208Fsrc3siCo) or siRNA directed towards dual oxidase1 (DUOX1) (208Fsrc3siDUOX1), but not in transformed 208Fsrc3 target cells transfected with siRNA directed towards NOX1 (208Fsrc3siNOX1) or non-transformed 208F target cells. Control measurements ensured that the dispersely seeded cells in this experiment did not show apoptosis induction above the background level at this time point.*

inhibited by the catalase inhibitor 3-AT ($p<0.001$) (Figure 1E and F). Apoptosis induction in oncogene-transformed cells and in tumor cells in the presence of 3-AT was inhibited by the POD inhibitor ABH, the HOCl scavenger taurine, the superoxide anion scavenger SOD and the hydroxyl radical scavenger mannitol ($p<0.001$). Therefore, in line with recently published work (26, 27, 29), these findings are explained by POD-catalyzed HOCl synthesis ($2O_2^{\bullet-} + 2H+ \rightarrow H_2O_2 + O_2$; $PODFe^{III} + H_2O_2 \rightarrow PODFe^{IV}=O^{\bullet+} + H_2O$; $PODFe^{IV}=O^{\bullet+} + Cl^- + H^+ \rightarrow PODFe^{III} + HOCl$) followed by the reaction $HOCl + O_2^{\bullet-} \rightarrow \bullet OH + Cl– + O_2$, whereby hydroxyl radicals seem to act as the final apoptosis inducers. When exogenous HOCl was directly added to cells from the three distinct steps of multistep carcinogenesis (Figure 1G), nontransformed cells did not react, whereas apoptosis was induced in transformed and in tumor cells in a superoxide anion-dependent reaction,

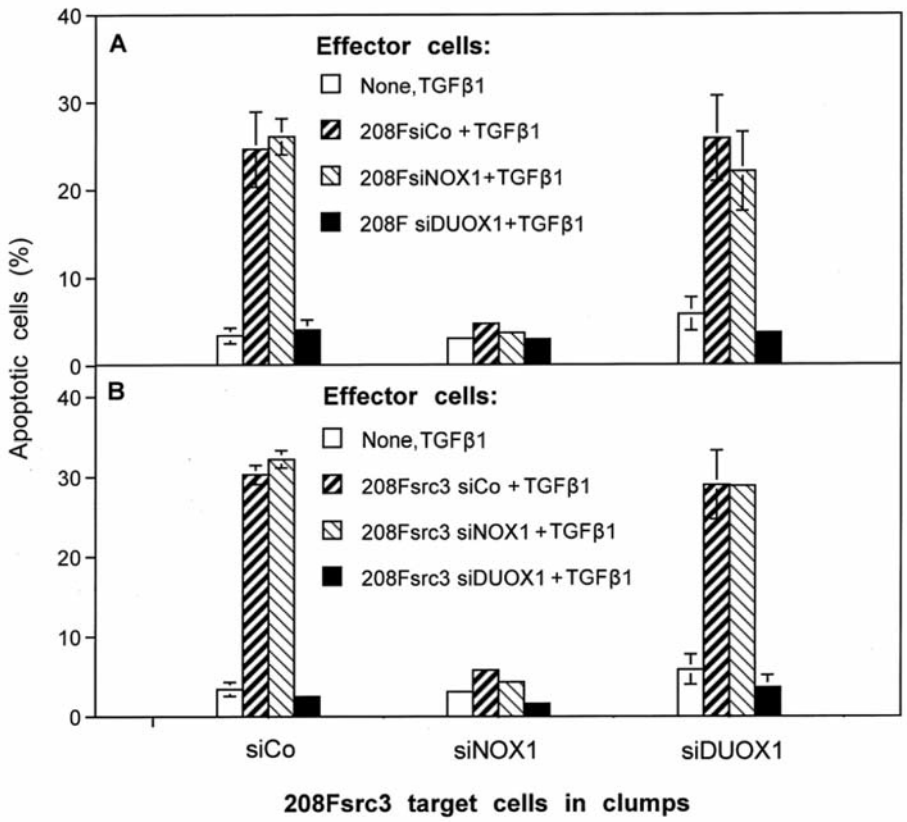

Figure 5. *The effector cell function in nontransformed and transformed cells is abrogated by siRNA directed against dual oxidase1 (DUOX1). In the presence of transforming growth factor-β1 (TGFβ1), transformed 208Fsrc3 target cells transfected with control siRNA (208Fsrc3siCo) or siRNA directed towards DUOX1 (208Fsrc3siDUOX1) showed substantial apoptosis induction when surrounded by nontransformed 208F effector cells transfected with siCO (208FsiCo) or towards NADPH oxidase 1 (208siNOX1) (A) or by transformed 208Fsrc3siCo or 208Fsrc3 siNOX1 effector cells. Transformed 208Fsrc3siNOX1 cells did not respond to effector cells. 208FsiDUOX1 effector cells (A) and 208Fsrc3siDUOX1 effector cells (B) did not induce apoptosis in 208Fsrc3siCo and 208Fsrc3siDUOX1 target cells.*

as shown by the inhibitory effect of SOD. These controls show that extracellular superoxide anion production by transformed cells and tumor cells determines their specific response to exogenous HOCl and that tumor cell catalase that prevents HOCl synthesis does not protect against exogenous HOCl.

Experimental dissection of target and effector cell functions involved in the HOCl signaling pathway. Previous work has shown that the target cell function i) is specific for transformed cells, ii) depends on the generation of extracellular superoxide anions and iii) requires a substantial local density of the cells (27). The effector function was initially studied in nontransformed cells (27), but has been shown to be active in non-transformed, transformed and tumor cells (23). This established knowledge was the basis for a cell culture system that allowed dissection of target and effector cell functions. This dissection of target and effector functions is illustrated in Figure 2. Transformed target cells were seeded as clumps of relatively low cell number, but high local density. They were further cultivated alone, or were surrounded by either non-transformed or transformed effector cells at high number, but low local density. Alternatively, MPO was added to the clumps. In addition, dispersely seeded transformed cells at low density but high number were cultivated in the presence of hydrogen peroxide-generating GOX. As essential control, clumps of non-transformed target cells were cultivated under the same conditions as their transformed counterparts. Figure 2F shows one representative example of this control, where clumps of non-transformed cells were surrounded by transformed effector cells. Figure 2 depicts the essential outcome of this experiment. The quantitative results obtained from this experiment are shown in Figure 3.

Clumps of transformed cells seeded alone exhibited low spontaneous apoptosis which was slightly enhanced by the addition of TGFβ1 (Figure 3A) but was not significantly different from apoptosis induction in clumps of

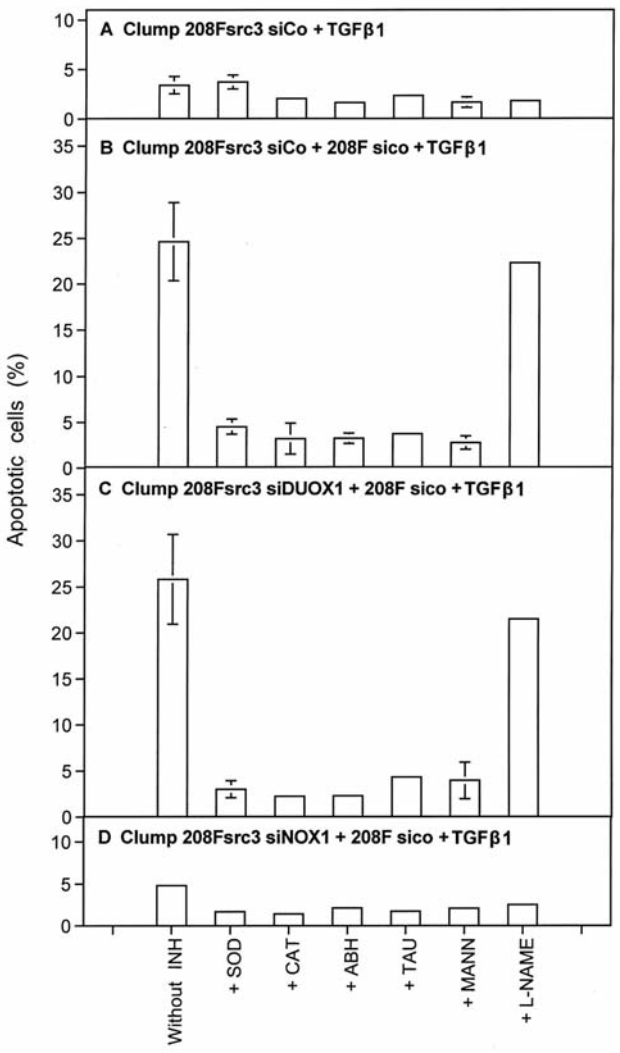

Figure 6. *HOCl signaling during intercellular induction of apoptosis. Clumps of transformed 208Fsrc3 target cells transfected with control siRNA (208Fsrc3siCo) in the presence of transforming growth factor-β1 (TGFβ1), but without surrounding effector cells (A) and 208Fsrc3 target cells transfected with siRNA directed towards NADPH oxidase1 (208Fsrc3siNOX1), surrounded by nontransformed 208FsiCo effector cells and TGFβ1 (D) did not show apoptosis induction, whereas clumps of 208Fsrc3siCo cells (B) or with siRNA directed towards DUOX1 (208Fsrc3siDUOX1) (C) surrounded by nontransformed 208FsiCo effector cells plus TGFβ1 showed substantial apoptosis induction. Apoptosis induction under B and C was inhibited when superoxide anions were scavenged by superoxide dismutase (SOD), hydrogen peroxide was decomposed by catalase, peroxidase was inhibited by aminobenzoyl hydrazide (ABH), HOCl was scavenged by taurine (TAU) and hydroxyl radicals were scavenged by mannitol (MANN), pointing to HOCl signaling as being the responsible mechanism. The NO synthase inhibitor L-NAME had no significant inhibitory effect.*

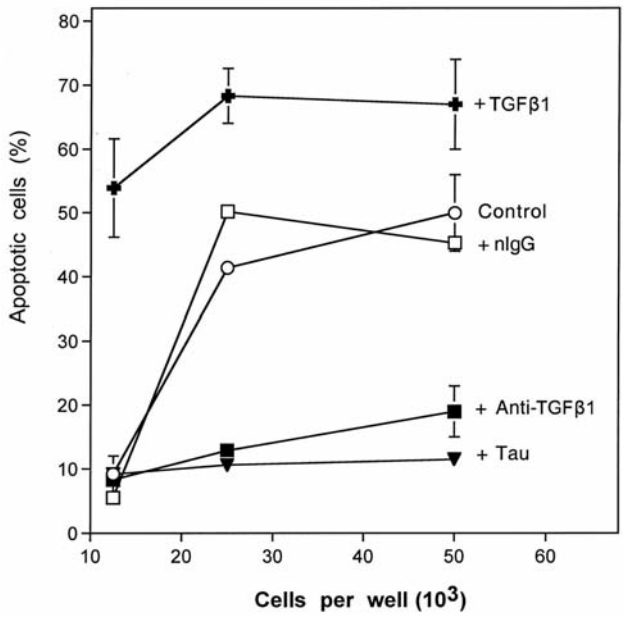

Figure 7. *The role of transforming growth factor-β1 (TGFβ1) for HOCl signaling. Transformed 208Fsrc3 cells showed density-dependent autocrine apoptosis induction that was enhanced by exogenous TGFβ1 and specifically inhibited by neutralizing anti-TGFβ1 or the HOCl scavenger taurine (TAU). These results point to the central role of TGFβ1 for HOCl signaling. They also show that cell-derived TGFβ1 contributes to HOCl signaling, although it was present at suboptimal concentrations under the conditions of our assays.*

nontransformed cells (p=0.03). Addition of MPO or the NO donor SNP caused marked apoptosis induction in clumps of transformed 208Fsrc3 cells (p<0.001), but had no effect on non-transformed 208F cells (Figure 3A). Addition of dispersely seeded nontransformed 208F or transformed 208Fsrc3 cells to assays containing clumps of transformed 208Fsrc3 cells caused apoptosis induction specifically in the clumps (p<0.001). This apoptosis induction was enhanced by the addition of TGFβ1 (p<0.001). Non-transformed target cells did not show apoptosis induction in the presence of dispersely seeded effector cells, independent of the presence of TGFβ1. These data demonstrate that optimal apoptosis induction in transformed cells that are seeded as clumps requires either the addition of defined signaling components such as MPO or SNP, or the presence of a surplus of dispersely seeded non-transformed or transformed effector cells plus TGFβ1. Transformed target cells require either the presence of sufficient MPO or an excess of dispersely seeded effector cells for apoptosis induction (Figure 3B). As the effect of the dispersely seeded effector cells was inhibited by the POD inhibitor ABH, POD released from the effector cells and acting in *trans* seemed to be responsible for the effector function observed.

Whereas added MPO or POD released from effector cells acts with the clump of target cells in the establishment of HOCl signaling, an increase in concentration of hydrogen peroxide

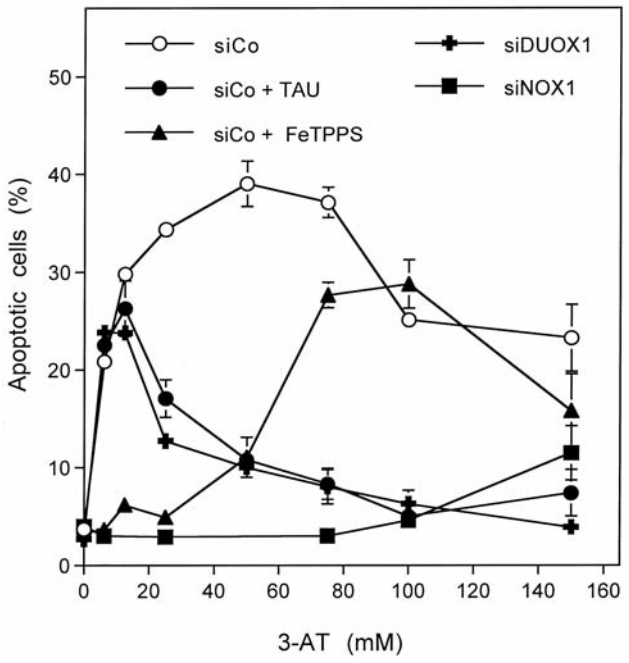

Figure 8. *Knockdown of NADPH oxidase1 (NOX1) and dual oxidase1 (DUOX1) inhibit HOCl-mediated autocrine apoptosis induction in MKN-45 tumor cells MKN-45 cells transfected with control siRNA (siCo) showed apoptosis induction in the mode of an optimum curve, dependent on the concentration of the catalase inhibitor 3-aminotriazole (3-AT) added to the assays. siNOX1-dependent knockdown of NOX1 completely abrogated 3-AT-mediated apoptosis induction, whereas siDUOX1-dependent knockdown of DUOX1 prevented apoptosis induction at concentrations of 3-AT between 25-150 mM, but not at lower concentrations of the inhibitor. This pattern of prevention of apoptosis induction by siDUOX1 correlated with inhibition by the HOCl scavenger taurine (TAU), indicating that siDUOX1 had specifically affected the HOCl signaling pathway, whereas the NO/peroxynitrite signaling pathway that was effective at lower concentrations of 3-AT (as confirmed by the inhibitory effect of the peroxynitrite decomposition catalyst FeTPPS) was not affected by siDUOX1.*

through addition of GOX did not affect the isolated clumps of target cells (Figure 3B). Dispersely seeded transformed effector cells surrounding the clump of transformed target cells did not exhibit apoptosis induction within the time of the experiment (Figure 3C). Addition of MPO had no effect on dispersely seeded transformed cells, whereas addition of GOX caused marked apoptosis induction ($p<0.001$).

Taken together, these initial data confirm that the target cell function is specific for transformed cells, whereas the effector function is independent of the transformed state. They also demonstrate that the clumps of transformed cells seem to lack POD required to establish ROS-dependent apoptosis signaling, whereas the dispersely seeded cells seem to generate a sufficient concentration of POD that acts in *trans* on the transformed target cells, but seem to lack sufficient hydrogen peroxide for their own apoptosis signaling.

The target cell function of transformed cells is defined by NOX1. The experimental system described in Figure 2 and 3 allowed us to specifically address the question regarding the nature of the superoxide anion-generating enzyme in transformed cells. Nontransformed 208F cells (transfected with control siRNA siCo) and transformed 208Fsrc3 cells (transfected either with siCO, siNOX1 or siDUOX1) were seeded as clumps 24 h after transfection. These clumps of target cells remained without addition of dispersely seeded effector cells or were combined with dispersely seeded 208FsiCo cells (Figure 4A) or 208Fsrc3siCo cells (Figure 4B). Parallel assays received TGFβ1 or did not. Some assays received the POD inhibitor ABH. The results shown in Figure 4 confirm that nontransformed 208FsiCo cells did not exhibit target cell function under any of the conditions applied. In contrast, transformed 208Fsrc3siCo cells seeded in clumps exhibited target cell function and died through apoptosis induction when they were co-cultured either with dispersely seeded nontransformed 208F or transformed 208Fsrc3 effector cells ($p<0.001$). Apoptosis induction in transformed target cells in the presence of dispersely seeded effector cells was completely blocked by ABH, indicating that a *trans*-acting POD was responsible for apoptosis-inducing signaling. Knockdown of *NOX1* in the target cells completely abrogated the target cell function of 208Fsrc3 cells ($p<0.001$), whereas treatment with irrelevant control siRNA or knockdown of DUOX1 had no effect on the target cell function.

These results define NOX1 as the central enzyme for the execution of the target cell function.

The effector function of transformed and nontransformed cells is defined by DUOX1. When clumps of transformed 208Fsrc3 siCo, 208Fsrc3 siNOX1 and 208Fsrc3 siDUOX1 target cells were overlaid with dispersely seeded nontransformed 208F or transformed 208Fsrc3 cells that had been pretreated either with siRNA, apoptosis induction was successful when clumps of target cells with functional NOX1 (*i.e.* siCo and siDUOX1) were co-cultured with siCo or siNOX1-transfected effector cells ($p<0.001$), but not when *DUOX1* had been knocked down in the effector cells (Figure 5).

This result shows that DUOX1, but not NOX1, is required for the effector function that acts in *trans* on target cells and confirms that NOX1, but not DUOX1 is necessary for the target cell function.

The interaction between transformed target cells and effector cells is due to the HOCl signaling pathway. Clumps of 208Fsrc3 siCo target cells did not exhibit apoptosis induction when seeded alone (Figure 5A). Addition of dispersely seeded nontransformed 208F cells plus TGFβ1 caused marked apoptosis induction in 208Fsrc3 siCo (Figure 5B) and 208Fsrc3 siDUOX1 (Figure 5C) ($p<0.001$), but not in 208Fsrc3siNOX1 target cells (Figure 5D). Effector cell-

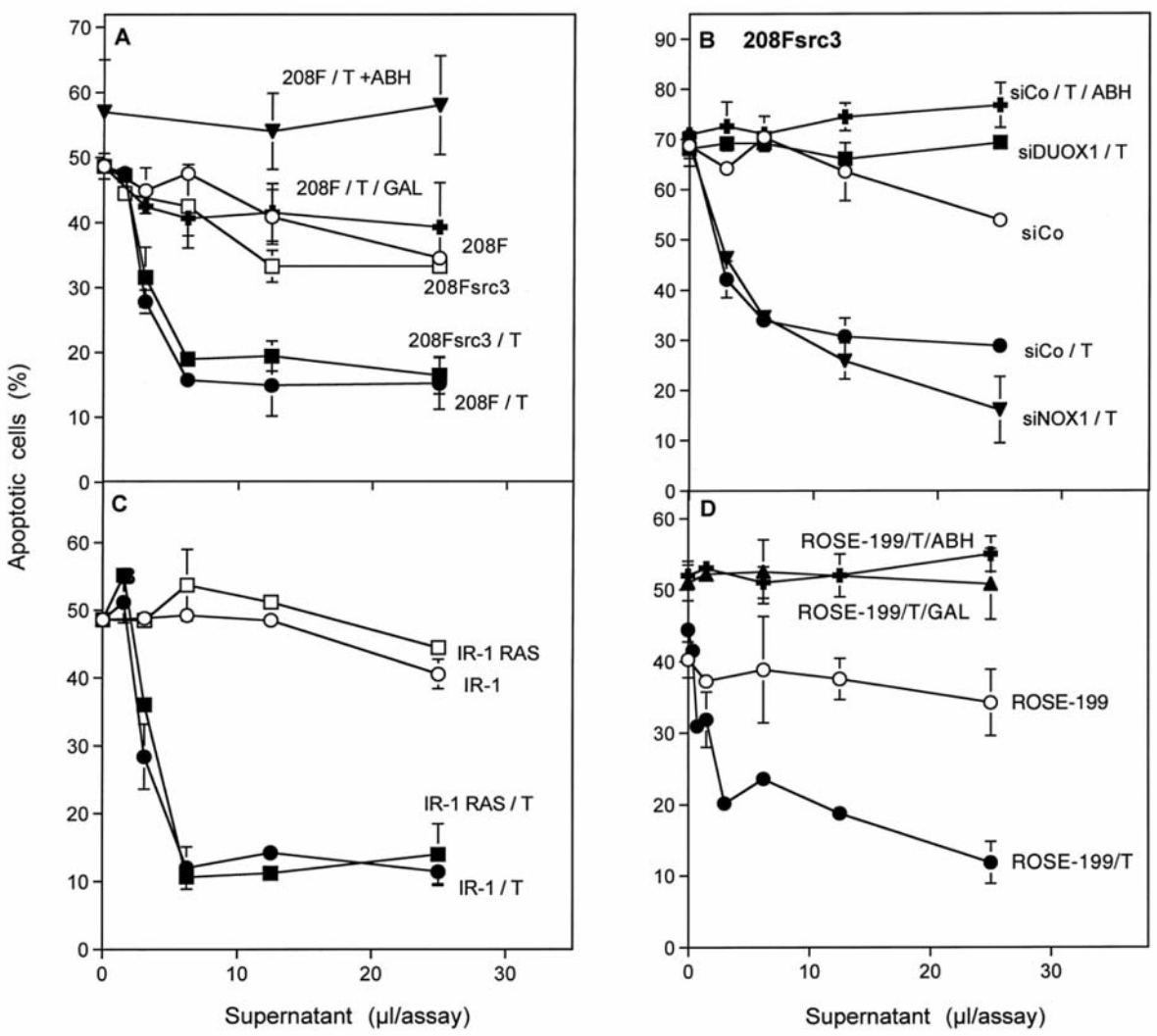

Figure 9. *Dual oxidase1 (DUOX1)-coded peroxidase (POD) is released by non-transformed and oncogene-transformed cells after stimulation with transforming growth factor-β1 (TGFβ1) and through the action of a matrix metalloproteinase. The test system responds to the presence of peroxidase in supernatants harvested from cells with a decrease in EUK-8-mediated apoptosis induction. This decrease is dependent on the volume of added supernatant (see Materials and Methods for details). A: POD release was found for TGFβ1-treated 208F (208F/T) and 208Fsrc3 cells (208Fsrc3/T). POD activity was assured through abrogation of the inhibitory effect through the peroxidase inhibitor aminobenzoyl hydrazide (ABH), exemplarily shown for the supernatant from TGFβ1-treated 208F cells (208/T + ABH) that was tested in the presence of ABH. The presence of the matrix metalloproteinase inhibitor galardin (GAL) during collection of the supernatants prevented POD release. B: POD was released from TGFβ1-treated 208Fsrc3 cells transfected with control siRNA (siCo/T), siRNA directed towards NADPH oxidase1 (NOX1) (siNOX1/T), but not from TGFβ1-treated 208Fsrc3 transfected with siRNA directed DUOX1 (siDUOX1/T) 24 h before the preparation of the samples. C: POD was released from TGFβ1-treated IR-1 cells (IR-1/T) and IR-1 cells with an induced Ras oncogene (IR-1 RAS/T. D: POD was released from TGFβ1-treated ROSE-199 epithelial cells (ROSE-199/T). The specificity of the reaction was assured by the abrogating effect of ABH on inhibition (ROSE-199/T/ABH).*

mediated apoptosis induction in 208Fsrc3 siCo and 208Fsrc3 siDUOX1 target cells was dependent on superoxide anions (as it was inhibited by SOD), hydrogen peroxide (as it was blocked by catalase), HOCl synthesis byPOD (as it was blocked by ABH and taurine), and hydroxyl radicals (as it was inhibited by mannitol) ($p<0.001$). Inhibition of NO synthase by L-NAME only had a minor effect.

This finding confirms that NOX1-dependent apoptosis induction in transformed target cells depends on the HOCl signaling pathway, which is defined by HOCl synthesis and subsequent HOCl/superoxide anion interaction, leading to apoptosis-inducing hydroxyl radicals. This result also confirms that the NOX domain of DUOX1 is not required for the target function.

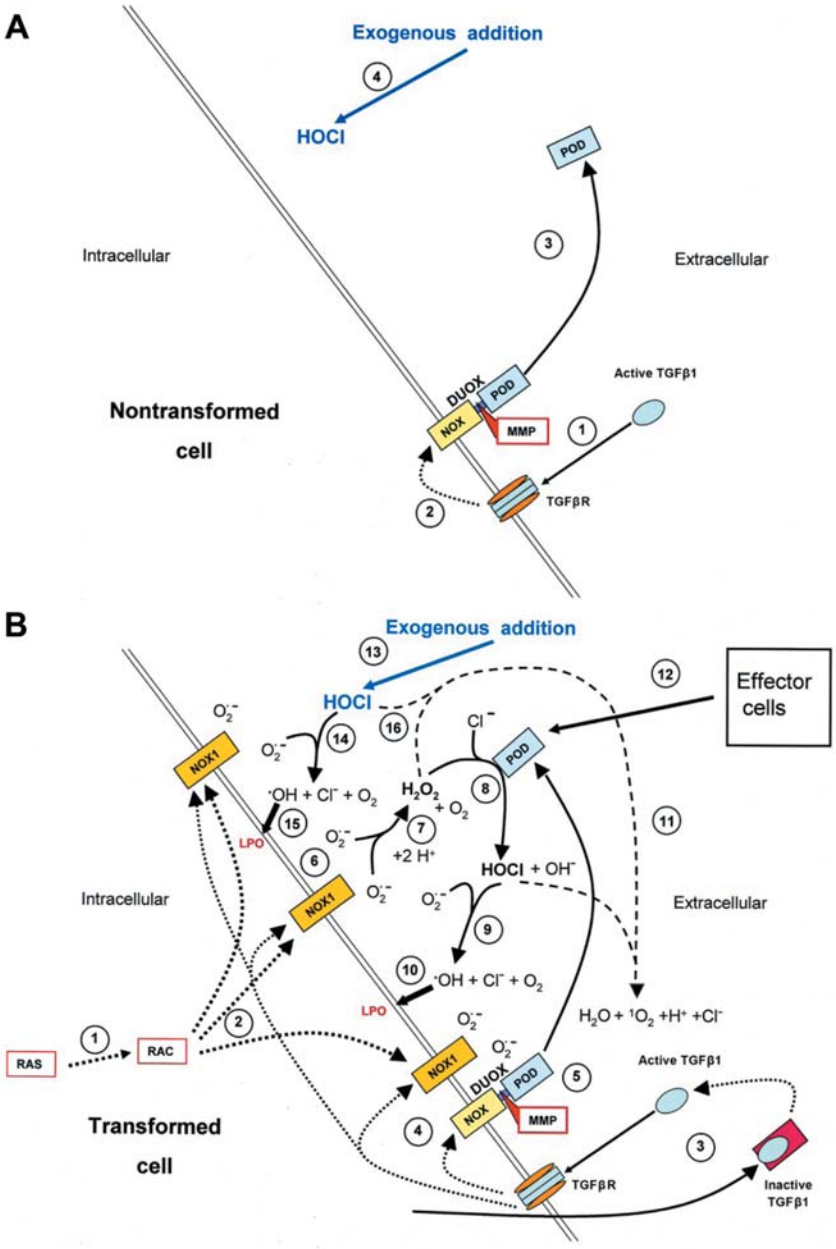

Figure 10. *The role of NADPH oxidase1 (NOX1) and dual oxidase1 (DUOX1) after transformation. A: Transforming growth factor-β1 (TGFβ1) (#1) stimulates the expression of DUOX (#2) and the release of the peroxidase (POD) domain of DUOX (#3) through the activity of matrix metalloproteinase (MMP). The residual DUOX-related NOX domain does not generate extracellular superoxide anions an as nontransformed cells do not express NOX1, they lack sustained production of extracellular superoxide anions. Therefore, they show no apoptotic response to exogenously added HOCl or NO (not shown in the Figure). Non-transformed cells lack the target cell function that is characteristic of malignant cells, but contribute to intercellular reactive oxygen species (ROS)-mediated signaling through POD. B: Transformed cells are controlled by oncogenes (shown here for the rat sarcoma oncogene (RAS), that controls RAS-related C3 botulinum toxin substrate (RAC), a member of the Rho family of small GTPases) (#1). RAC activates NOX1 (#2). The cells release inactive TGFβ1 that is activated (#3) and which stimulates the TGFβ receptor. Stimulation of TGFβ receptor causes induction of NOX1 activity (#4) (40) and induction of DUOX (#4), whose POD domain is released through the action of matrix metalloproteinases (MMP) (#5). Superoxide anions generated by NOX1 (#6) allow dismutation to H_2O_2 (#7) which is used by POD as substrate for the generation of HOCl (#8). HOCl then interacts with superoxide anions (#9), leading to the formation of hydroxyl radicals that cause lipid peroxidation (LPO) in the cell membrane and subsequent apoptosis induction (#10). When there is a relative excess of H_2O_2 compared to POD, HOCl may be consumed by H_2O_2 (#11). Neighbouring effector cells (nontransformed or transformed) can contribute to the enhancement of HOCl signaling through supplying POD (#12). Exogenously added HOCl (#13) interacts with NOX1-derived superoxide anions (#14) and forms apoptosis-inducing hydroxyl radicals (#15) or may be (partially) consumed by excess H_2O_2 (#16). When the POD domain of DUOX remains located at the membrane (after inhibition of MMP; not shown in this Figure), it decomposes exogenously added HOCl through the reverse reaction of POD, as outlined in our companion article in this issue.*

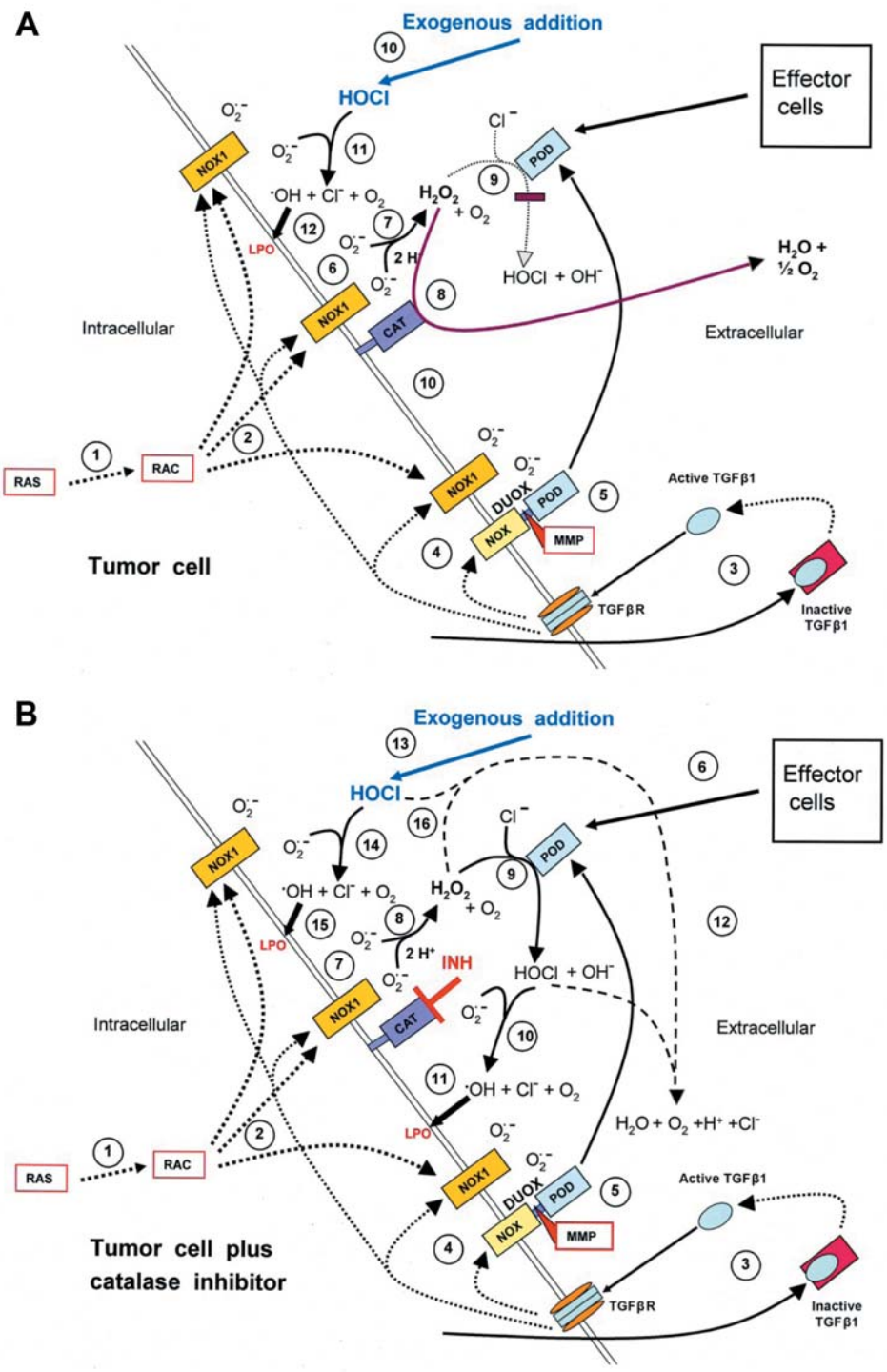

Figure 11. *The role of NADPH oxidase1 (NOX1) and dual oxidase1 (DUOX1) after tumor progression. A: Tumor cells are protected against HOCl signaling through membrane-associated catalase (23, 25). Therefore, H_2O_2 generated through dismutation of NOX1-derived superoxide anions (#7) is decomposed by the tumor cell-specific catalase and cannot foster HOCl synthesis (#9). In this way, tumor cells are protected against HOCl signaling, despite possessing active NOX1 and DUOX. Tumor cell catalase does not protect against apoptosis induction by exogenous HOCl (#10-12), it rather enhances this reaction as decomposition of H_2O_2 by catalase prevents consumption of HOCl by H_2O_2. B: Inhibition of membrane-associated catalase of tumor cells by low molecular weight inhibitors or antibodies, or its inactivation by singlet oxygen (29) abrogate decomposition of H_2O_2 and thus reactivate intercellular HOCl-dependent apoptosis signaling (#1-11). Apoptosis induction by exogenously added HOCl (#13-15) is reduced under these conditions, due to the consumption of HOCl by H_2O_2 (28).*

The role of TGFβ1 during ROS-mediated apoptosis induction in transformed cells. Transformed cells seeded at increasing cell density in a small volume exhibited apoptosis induction dependent on their density (Figure 7) ($p<0.001$). Apoptosis induction was enhanced by exogenous TGFβ1 ($p<0.001$), with the most prominent effect being apparent on the cells with the lowest cell density. The presence of neutralizing antibody directed against TGFβ1, but not control IgG, prevented apoptosis induction ($p<0.001$), pointing to the central role of endogenous TGFβ1 for the induction of ROS-mediated apoptosis induction.

Intercellular ROS-mediated apoptosis signaling in MKN-45 human gastric carcinoma cells after inhibition of catalase. MKN-45 cells (like all other bona fide tumor cells tested so far) are protected by membrane-associated catalase against intercellular ROS-mediated apoptosis signaling (25, 26, 50, 51). It has been shown that gradual inhibition of its protective catalase first reactivates NO/peroxynitrite signaling which is followed by HOCl signaling (25). For the evaluation of the role of NOX1 and DUOX1 in apoptosis induction in MKN-45 cells, the cells were transfected either with siCo or siNOX1 or siDUOX. After 24 hours, the cells were seeded in fresh medium and cultivated in the presence of increasing concentrations of the catalase inhibitor 3-AT. SiCo-treated cells also received the HOCl scavenger taurine and the peroxynitrite decomposition catalyst FeTPPS to discriminate between NO/peroxynitrite and HOCl signaling. Inhibition of catalase by increasing concentrations of 3-AT caused apoptosis induction ($p<0.001$) in siCo-transfected cells in the mode of the characteristic optimum curve (25) (Figure 8). At lower 3-AT concentrations, NO/peroxynitrite signaling seemed to be dominant, whereas at higher concentrations of 3-AT, apoptosis was controlled through the HOCl pathway, as deduced from the complimentary pattern of action of the inhibitors ($p<0.001$). Knockdown of *NOX1* prevented 3-AT-mediated apoptosis induction completely, confirming the central role for NOX1-generated superoxide anions for intercellular signaling through the NO/peroxynitrite and the HOCl signaling pathway. The knockdown of DUOX1 had the same effect as scavenging HOCl by taurine, indicating the role of DUOX1 for HOCl signaling in tumor cells after catalase inhibition ($p<0.001$).

DUOX1-related POD is released from nontransformed and transformed cells upon stimulation with TGFβ1. Measurement of POD activity in the supernatant from non-transformed 208F or IR1 cells and *Src* or *RAS* oncogene-transformed 208Fsrc3 or IR-1 RAS cells in a novel competition test for the detection of HOCl-generating PODs showed that nontransformed as well as transformed cells released similar amounts of POD after TGFβ1 treatment (Figure 9A-C). The presence of active POD in the supernatant was reflected in a decrease of apoptosis induction dependent on the volume of supernatant added. The release was abrogated ($p<0.001$) when the matrix metalloprotein inhibitor galardin was added to the cells during collection of supernatant (Figure 9A), indicating that POD was removed from the cell surface through action of matrix metalloproteases. The use of 208F cells with constitutive Src oncogene expression or an inducible *RAS* oncogene demonstrated that POD release is not affected by the state of transformation, as control cells and cells with induced oncogenes exhibited the same degree of POD release (Figure 9 A-C). The release of POD was prevented by preceding knockdown of *DUOX1* ($p<0.001$), but not by knockdown of *NOX1* (Figure 9B). Release of POD leading to reduced apoptosis, triggered by TGFβ1 and mediated by matrix metalloproteases, was also seen in epithelial cells (Figure 9 D).

Discussion

Our data confirm that the malignant phenotype, characterized by NOX-dependent extracellular superoxide anion generation (17-25), is essential for apoptosis induction by exogenous HOCl and for the establishment of autocrine apoptosis-inducing HOCl signaling. As seen by the effects of the inhibitors applied, selective apoptosis induction in malignant cells by HOCl in the micromolar concentration range is due to the formation of apoptosis-inducing hydroxyl radicals after HOCl/superoxide anion interaction. As nontransformed cells lack extracellular superoxide anion generation, they are not affected by HOCl in the micromolar concentration range. Membrane-associated catalase of tumor cells is supporting apoptosis induction by exogenous HOCl as it prevents the consumption reaction between HOCl and cell-derived hydrogen peroxide (28).

Autocrine HOCl signaling is established by homogenous populations of transformed cells that utilize their extracellularly produced superoxide anions as source for the formation of hydrogen peroxide, the substrate for the HOCl-generating POD and for the final step of HOCl/superoxide anion interaction that generates the apoptosis-inducing hydroxyl radicals as described above (Figure 10B). Efficient autocrine HOCl signaling requires a sufficiently high cell density to ensure optimal dismutation of superoxide anions to hydrogen peroxide and a sufficiently high cell number to ensure optimal POD concentration. Non-transformed cell populations cannot establish autocrine HOCl signaling as they lack superoxide anion production (Figure 10A). Tumor cells are resistant to autocrine HOCl signaling as their membrane-associated catalase decomposes hydrogen peroxide and thus prevents HOCl synthesis (Figure 11A). However, inactivation of tumor cell protective catalase reactivates autocrine HOCl signaling (23, 25, 48, 50, 51) (Figure 11B).

The dissection of the experimental system for the study of intercellular ROS signaling allowed a clear-cut differentiation

between the target cell function and the effector function. As depicted in Figure 2, the target function requires high local cell density and is specific for cells with the malignant phenotype, *i.e.* cells that generate extracellular superoxide anions (and subsequently hydrogen peroxide), whereas the effector function can be exerted by malignant and nontransformed cells with equal efficiency. The effector cell function is defined as supply with sufficient POD to allow HOCl synthesis specifically in the vicinity of the target cells, followed by hydroxyl radical generation driven by target cell-derived superoxide anions. The effector function can be mimicked by the addition of MPO. The target function cannot be mimicked, as it requires superoxide anion generation specifically at the membrane of the target cells. However, transformed cells seeded at sub-optimal density but high cell number can be induced to die by apoptosis through HOCl signaling when their suboptimal generation of hydrogen peroxide is supported by addition of hydrogen peroxide-generating GOX.

The dissection of the system allowed us to define the molecular partners that are involved in the target and effector function. Membrane-associated NOX1 defines the target function and its knockdown abolishes the sensitivity of the transformed cells to HOCl signaling, but does not affect their effector function. DUOX1, which is composed of an extracellular NOX domain and a POD domain (30, 34), does not contribute significantly to superoxide anion production, as knockdown of *DUOX1* has no effect on the target function of transformed cells. Furthermore, nontransformed cells that express DUOX1 are not affected by exogenous NO or HOCl that required extracellular superoxide anion generation for apoptosis induction (26, 27, 44). However, knockdown of *DUOX1* completely abolishes the effector function of transformed and nontransformed cells, indicating that the POD domain of DUOX1 or a closely-related protein represents the enzyme that is responsible for HOCl synthesis during HOCl signaling. In line with this conclusion, inhibition of POD activity by ABH had the same effect as knockdown of *DUOX1*.

Our data show that DUOX1 seems to act in trans, *i.e.* it must be released from the effector cells and establish a sufficiently high POD concentration in the vicinity of the hydrogen peroxide- and superoxide anion-generating target cells. Direct demonstration of POD activity in the supernatant of TGFβ1-treated non-transformed and transformed cells is in line with this conclusion. The lack of POD release from cells with siRNA-mediated knockdown of *DUOX1* points to DUOX1 or a closely related protein as being the responsible POD acting during HOCl-mediated intercellular apoptosis induction and the abrogation of release of POD in the presence of the matrix metalloproteinase inhibitor galardin indicates that the release of the DUOX-coded POD is catalyzed by matrix metalloproteinase. Our companion article in this issue presents data that show that the release of the POD domain of DUOX1 through matrix metalloproteinase is not a prerequisite for the activity of the POD.

MPO, eosinophilic POD and lactoperoxidase have usually been regarded as the only POD s with the potential to generate HOCl, in addition to their classical POD function. Vascular peroxidase1 (VPO1), a heme POD related to the POD domain of DUOX (52) was also recently shown to have the potential to generate HOCl (53). Due to their expression in highly specialized cells, these enzymes were not candidates for explaining the POD function in apoptosis-inducing HOCl signaling that has been found in cell systems of many different tissues (29). The list of mammalian enzymes with the potential to generate HOCl is now extended by DUOX, which exhibits HOCl synthesis in the presence of an optimal hydrogen peroxide concentration and that strikingly resembles MPO in all parameters tested. DUOX activity explains establishment of HOCl signaling in tumor cells from many different tissues. As its POD domain reaches into the extracellular space, there is a good chance that the specific action of proteases causes release of this domain and thus enables the observed action of *DUOX* in trans. Our companion article addresses this important aspect. HOCl-mediated apoptosis signaling after catalase inhibition has been seen in the human glioblastoma A172 cells, the lung carcinoma SKMES-1, A549 and LCLC-103H cells, melanoma IPC-298 and MEL-JUSO cells, cervical carcinoma SISO cells and SIHA, pancreatic tumor PATU 8902 cells, colon carcinoma HT29 cells and others (Bauer, unpublished data). siRNA-mediated knockdown of *DUOX1*, for example in PATU 8902, SISO, SIHA cells caused complete abrogation of HOCl-mediated apoptosis induction (Bauer, unpublished data). The release of DUOX (most likely its POD domain) explains the effector function of many cells and the action in *trans* of the effector function involved in HOCl signaling. Our finding is in agreement with the pioneering work by Chen *et al*. (54), who have utilized a novel approach to detect HOCl and have linked HOCl synthesis to DUOX activity in the gut of *Drosophila*.

Acknowledgements

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