

FOXO3a: a novel player in thyroid carcinogenesis?

Stefan Karger*, Carl Weidinger*, Kerstin Krause, Sien-Yi Sheu¹, Thomas Aigner², Oliver Gimm³, Kurt-Werner Schmid¹, Henning Dralle³ and Dagmar Fuhrer

Division of Endocrinology and Diabetes, Department of Internal Medicine, University of Leipzig, Ph.-Rosenthal-Street 27, 04103 Leipzig, Germany

¹Institute of Pathology and Neuropathology, University of Duisburg-Essen, Essen, Germany

²Institute of Pathology, University of Leipzig, Leipzig, Germany

³Department of Surgery, University of Halle-Wittenberg, Halle, Germany

(Correspondence should be addressed to D Fuhrer; Email: fued@medizin.uni-leipzig.de)

*(S Karger and C Weidinger contributed equally to this study)

Abstract

The forkhead box transcription factor FOXO3a has recently been identified as central mediator of the cellular response to oxidative stress inducing cell cycle arrest or apoptosis. The aim of our study was to investigate the regulation of FOXO3a in the thyroid and to determine whether alterations in FOXO3a activity occur in thyroid carcinogenesis. *In vitro*, we demonstrate that FOXO3a activity is negatively regulated by the PI3K/Akt cascade promoting increased phosphorylation and cytoplasmatic accumulation of FOXO3a with decreased transcription of the target genes p27kip (*CDKN1B*) and Bim (*BCL2L11*), but increased expression of *GADD45A*. By contrast, we show that H₂O₂ exposure activates FOXO3a in thyrocytes with JNK (MAPK8)-mediated nuclear accumulation of FOXO3a and increased expression of the cell cycle arrest genes p27kip and *GADD45A*. *In vivo*, we observed a marked cytoplasmatic accumulation of FOXO3a in differentiated thyroid cancers versus an exclusive nuclear accumulation in follicular adenoma and normal thyroid tissue. Moreover, this cytosolic accumulation of FOXO3a correlated with an increased phospho-Akt expression in thyroid malignancies and was accompanied by decreased expression of the FOXO targets p27kip and Bim and an increase in *GADD45A* mRNA expression in the thyroid cancers. Our data suggest FOXO3a as a novel player of cellular stress response in the thyroid, mediating the thyrocyte's fate either to survive or to undergo apoptosis. Furthermore, PI3K-dependent FOXO3a inactivation may be a novel pathomechanism for the escape from apoptosis in thyroid cancer cells, in particular in follicular thyroid carcinoma.

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Introduction

Growth factor-induced cellular proliferation depends on specific survival signaling (Birchenkamp & Coffey 2003, van der Horst & Burgering 2007). In the absence of survival signals, cells do not proliferate but rather undergo apoptosis. Mutations in regulatory cell cycle genes may disturb the cellular homeostasis existing between proliferation and apoptosis, and cells may escape controlled growth (Bartkova *et al.* 2005, Essers *et al.* 2005).

Recently, the subclass O of forkhead transcription factors (FOXO) have been linked to the regulation of

cellular proliferation and apoptosis: FOXOs promote tumor suppression by the induction of proteins mediating cell cycle arrest, apoptosis, and DNA damage repair. In addition, they regulate cellular longevity by exerting resistance to oxidative stress and metabolic adaptation to nutritional deprivation (Essers *et al.* 2005, Greer *et al.* 2007). To date, four different isoforms of FOXOs are known in mammals and are termed FOXO1, FOXO3a, FOXO4, and FOXO6 (Huang & Tindall 2006).

FOXO3a is localized in the nucleus, where it activates or represses the transcription of target genes. Upon stimulation with growth factors FOXO3a is

phosphorylated, which accelerates the nuclear exclusion of FOXO3a, thereby inhibiting its ability to bind DNA. The nuclear export of FOXO3a is mediated by 14-3-3 proteins and exportins, e.g., CRM1, which also prevent the nuclear reimport of FOXO3a (Birkenkamp & Coffey 2003, van der Heide et al. 2004). Besides phosphorylation, FOXO proteins can undergo further posttranslational modifications such as acetylation and deacetylation. These steps are mandatory for the specificity of FOXO protein activity, which is reflected by distinct target gene expression such as Bim (*BCL2L11*), p27kip (*CDKN1B*), and *GADD45A* (Brunet et al. 2004). Posttranslational inactivation of FOXO3a with combined nuclear exclusion and target gene dysregulation has been observed in several types of cancer. Thus, dysregulation of the FOXO3a target genes Bim, p27kip, and *GADD45A* with combined cytoplasmic FOXO3a has been found in breast, lung, and prostate cancers. Bim has been described to be one of the major target genes of FOXO3a and is critical for the release of mitochondrial cytochrome *c*, and therefore of pivotal importance for the apoptotic program (Dijkers et al. 2000a). The FOXO target p27kip inhibits cyclin E/CDK2 complexes at the G1/S boundary of the cell cycle, which drives cells from growth into the state of cellular quiescence (Dijkers et al. 2000b, Stahl et al. 2002, Lee et al. 2006, van der Horst & Burgering 2007). *GADD45A* plays an important role in the control of cell cycle checkpoints and DNA repair (Hildesheim et al. 2002, Gupta et al. 2006, Tront et al. 2006). For example, *GADD45A* can block the formation of cyclin/CDK complexes at the G2/M-phase boundary, resulting in growth arrest. Furthermore, *GADD45A* was described to act as a base excision protein in response to ionizing irradiation.

Thyroid epithelial neoplasia represents a unique entity, since tumors with very different functional and histological characteristics, malignant potential, and degrees of differentiation can arise from a single ancestor cell (Fagin 2002). The event determining the different phenotypes, e.g., hyper- and hypo-functioning thyroid nodules, and benign and malignant thyroid tumors, appears to differ from the point of the clonal initiation. Specific genetic alterations involved in thyroid tumorigenesis have been identified mainly in malignant thyroid tumors, notably RET-PTC transgenes, BRAF V600E mutations in papillary thyroid cancers, PAX8-PPARG rearrangements, and retrovirus-associated DNA sequences (RAS) mutations in the subsets of follicular thyroid cancers and RET-*proto-oncogene* mutations in medullary thyroid cancers (Kroll et al. 2000, Fagin 2002, Nikiforova et al. 2003, Santoro et al. 2004).

Presently, a pivotal role of oxidative stress is discussed in thyroid physiology and disease (Krause et al. 2007b, Song et al. 2007), and has been found to be consistent with an increase in mutagenesis in the thyroid (Maier et al. 2006). In addition, we have recently observed increased levels of oxidative DNA damage in thyroid cancers, notably in follicular thyroid carcinoma (FTC; Karger S, et al. unpublished observations).

In view of the critical role that FOXOs play in oxidative stress defense, it might be possible that alterations of FOXO function by inhibition of FOXO activity or of its transcriptional activity may result in increased oxidative stress and an imbalance between proliferation and apoptosis. Therefore, the aim of this paper was to study the regulation of FOXO3a in the thyroid and to investigate FOXO3a expression and function in benign and malignant thyroid tumors.

Materials and methods

Thyroid samples

Duplicates of 22 hypofunctional follicular adenomas (FA), 22 corresponding normal thyroid tissues of the same patient normal tissue (NT), 14 FTC, and 10 classic PTC were studied for mRNA expression of *FOXO3a*, p27kip, Bim, and *GADD45A*. For immunohistochemical analysis of both FOXO3a and phospho-Akt (pAkt) protein expression, paraffin-embedded tissue sections from 65 FA, 68 FTC, and 63 PTC with adjacent normal thyroid tissue and 19 NT were used. Thyroid samples were obtained from the Department of Surgery, University of Halle-Wittenberg, Halle, Germany, local Leipzig hospitals and the Institute of Pathology and Neuropathology, University of Duisburg-Essen, Essen, Germany. Patient data and clinical characteristics are available upon request. Informed consent was obtained from all patients and the local ethics committee approved the study.

Cell culture

Follicular rat thyroid cells (FRTL-5 cells (Ambesi-Impimato et al. 1980); kindly provided by Prof. Di Lauro, Naples) were split into 6-well plates (3×10^5 /well) and were cultured in a 2:1:1 mixture of DMEM:Ham's F12:MCDB104 (all from Gibco Life Technologies) supplemented with 5% newborn calf serum (NCS; Gibco Life Technologies), 10 µg/ml insulin, 0.4 µg/ml hydrocortisone (Calbiochem, San Diego, CA, USA), 45 µg/ml ascorbic acid (Sigma), 5 µg/ml transferrin (Calbiochem), and 5 mU/ml bovine TSH (Sigma) until 70% confluence.

Prior to TSH, insulin, and NCS stimulation, FRTL-5 cells were starved for 48 h in the medium containing 0.2% serum but no TSH and insulin. Afterward, the cells were incubated in the presence of either 10 mU/ml TSH, 100 ng/ml insulin, or 5% NCS for 8 h. For controls, starving medium was changed and the cells remained in fresh starving. After the incubation period, the cells were washed with ice-cold PBS and were shock frozen in liquid nitrogen and stored at -80°C until further use.

For stimulation or inhibition of protein kinases, the cells were split into 6-well plates as described above and were grown in insulin and NCS containing growth media until 70% confluence. Then, either 10 mU/ml TSH, 10 μM forskolin (all from Sigma–Aldrich), 10 μM LY294002 (PI3K inhibitor), 1 μM PMA (PKC activator), or 20 μM GF10923X (PKC inhibitor; all from Calbiochem) were directly added into growth media, whereas controls were treated with dimethyl sulfoxide (DMSO) only. After stimulation, the cells were washed twice with PBS and were shock frozen in liquid nitrogen and stored at -80°C .

RNA extraction and RT-PCR

RNA was isolated from snap-frozen thyroid tissue using TRIzol reagent (Invitrogen). One microgram of RNA per sample was reverse transcribed in a final mixture of $5\times$ first-strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl_2 ; Gibco BRL), 0.5 mM dNTPs, 5 mM DTT (Gibco BRL), 15 U Prime RNase inhibitor (PeqLab, Erlangen, Germany), 0.5 μg random hexamer primers, and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL). Reverse transcription was performed at 37°C for 60 min and 94°C for 5 min.

Quantitative real-time PCR

Real-time PCR was performed using the LightCycler System (Roche). Exon/intron spanning primers were designed to quantitatively calculate human and rat *FOXO3a*, *p27kip*, *GADD45A*, and *Bim* mRNA expression (primers and PCR conditions available on request). The mRNA expression of the housekeeping protein β -actin and ribosomal rs6 protein were used for normalization of human samples and for standardization of FRTL-5 cells respectively (Eszlinger *et al.* 2005, Krause *et al.* 2007a,b).

To quantify changes in mRNA expression patterns, real-time PCR was performed using the LightCycler DNA Master SYBR Green I kit (Roche), as described previously (Krause *et al.* 2007a,b). Fold upregulation or downregulation of mRNA expression was calculated

as follows: $n=2(\text{threshold cycle of target gene NT-DT})/2(\text{threshold cycle } \beta\text{-actin NT-DT})$. ‘Diseased tissue’ (DT) corresponds to FA, FTC, and PTC, whereas ‘normal tissue’ corresponds to the surrounding tissue of FA. The Mann–Whitney *U*-test within the SPSS 11.5 was applied to calculate the statistical significance of differences in the mRNA expression of the respective genes between thyroid tissues (Fuhrer *et al.* 2005, Krause *et al.* 2007a,b).

Confocal laser scanning microscopy

FRTL-5 cells were grown on coverslips (1×10^5 /well). Experiments were performed as described under cell culture. Subsequently, the cells were permeabilized and fixed with 0.01% Triton X-100 in 2% paraformaldehyde/PBS (all from Sigma–Aldrich) for 20 min on ice. Afterward, the cells were washed three times with PBS followed by incubation with the primary antibodies overnight at 4°C with 0.1% BSA in PBS (FOXO3a, 1:1000; anti-phospho-FOXO3a, 1:1000). After washing the cells three times with PBS, the cells were incubated for 1 h with the secondary anti-rabbit Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR, USA) diluted at 1:1000 in PBS. Confocal analysis was performed on a confocal laser scanning system (TCS SP2; Leica, Wetzlar, Germany) attached to a microscope (100 \times oil immersion lens; PL Fluotar 1.3; Leica). Optical sections (0.45 μm) with approximately 150 cells were investigated.

Western blot analysis

Western blot analysis was performed using antibodies against FOXO3a (Cell Signaling, Charlottesville, VA, USA) and the phosphorylated form (Thr32) of FOXO3a, which represents the Akt phosphorylation site (van der Heide *et al.* 2004; Upstate Biotechnology, Lake Placid, NY, USA). Cells were lysed in buffer containing 10 mM Tris, 400 mM NaCl, 1 mM EDTA, and 0.1% NP-40 (all from Sigma). Fifty micrograms of protein were separated on 10% SDS gels followed by semi-dry western blotting. Subsequently, membranes were blocked with 5% BSA in TBST (50 mM Tris–HCl (pH 7.5) and 150 mM NaCl containing 0.05% Tween 20). Blots were probed overnight with the following dilutions of antibodies in 5% BSA containing TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween-20): anti-FOXO3a (1:1000), anti-phospho-FOXO3a (1:750), anti-phospho-JNK (*MAPK8*) (1:1000), and anti- β -actin (1:750). After incubation with a secondary anti-rabbit antibody coupled to HRP (Cell Signaling), immunocomplexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Subcellular fractionation

Subcellular fractionation was performed as described by Ni *et al.* (2007). Briefly, 3×10^5 FRTL-5 cells were split into Petri dishes and grown to confluence. Subsequently, $400 \mu\text{M}$ H_2O_2 were added to full growth media (containing insulin, TSH, and NCS) and the cells were incubated for 4 h. No hydrogen peroxide was added to the controls. Afterward, the cells were washed twice with ice-cold PBS, were shock frozen in liquid nitrogen, and stored at -80°C . The cells were then lysed in $250 \mu\text{l}$ ice-cold lysis buffer containing protease/phosphatase inhibitors and centrifuged at $3000 g$ for 10 min. The supernatant (containing the cytosolic protein fraction) was removed and mixed with $250 \mu\text{l}$ $2 \times$ SDS sample buffer. The remaining pellet with the nuclear protein fraction was suspended in $500 \mu\text{l}$ $1 \times$ SDS sample buffer. Equal volumes of the cytosolic and nuclear fraction were used for western blot analysis.

Immunohistochemistry

Paraffin-embedded tissue sections ($2 \mu\text{m}$) were deparaffinized in xylol and subsequently pretreated in a microwave oven in 0.1 M citrate buffer (pH 6) to allow antigen retrieval. For immunodetection, the LSAB + System (DAKO Cytomation, Hamburg, Germany) was used, according to the manufacturer's description. Briefly, slides were incubated with 3% H_2O_2 for 30 min followed by three washing steps with PBS including 1% BSA (PBS/BSA; Sigma). Subsequently, unspecific binding was blocked for 30 min followed by treatment with a) polyclonal rabbit FOXO3a antibody and b) monoclonal rabbit phospho-Akt antibody (both diluted 1:500 in PBS; Cell Signaling). After incubation with a biotinylated secondary goat anti-rabbit antibody, the immunocomplexes were detected by streptavidin peroxidase and diaminobenzidine. The sections were counterstained with hemalaun and mounted on AquaTex (Merck, Darmstadt, Germany). Positive controls were performed with tissue sections from small intestine (FOXO3a) and colon cancer (phospho-Akt) respectively.

Analysis of FOXO3a and pAkt protein staining was done by two independent investigators (SK/DF). Semiquantitative analysis of FOXO3a and pAkt was performed using the percentage of positively stained thyrocytes/ten randomly selected sections/slide ($200 \times$ magnification, duplicate). The staining index was calculated for each tissue sample and expressed as follows: 0=0% staining, 1 \geq 25% staining, 2=25–50% staining, 3=50–75% staining, and 4 \geq 75% staining.

Results

Effects of thyroid-specific growth factors and signaling cascades on FOXO3a function

Differential modulation of FOXO3a localization by TSH and insulin

In the thyroid, three different pathways regulate thyrocyte proliferation and differentiation: the TSH/cAMP/PKA, the insulin/PI3K/Akt, and the PKC signaling pathway (Roger & Dumont 1984, Medina & Santisteban 2000, Kimura *et al.* 2001).

In the first set of experiments, we analyzed the influence of TSH and insulin on FOXO3a function. To this aim, differentiated rat FRTL-5 cells were starved for 48 h (0.2% serum, without TSH and insulin). Subsequently, the cells were incubated with either 10 mU/ml TSH, 100 ng insulin, 5% serum, or full growth media (containing serum, TSH and insulin) for 8 h. Western blot analysis and confocal microscopy were applied to investigate FOXO3a activation.

Compared with FRTL-5, the cells kept in normal growth media, 48 h of starvation led to a decrease in FOXO3a phosphorylation and to strong nuclear accumulation of FOXO3a (Fig. 1). In the following experiments, this starvation condition was used as a control.

Subsequent addition of 10 mU/ml TSH did not change phosphorylation of the insulin-PI3K/Akt phosphorylation site Thr32, and calization compared with starved cells, but resulted in a marginal increase in total FOXO3a (Fig. 1B). By contrast, insulin and serum strongly induced FOXO3a Thr32 phosphorylation and nuclear exclusion of FOXO3a. The presence of all growth factors in the full growth media exerted an additive effect on FOXO3a phosphorylation (Fig. 1A and B).

Differential modulation of FOXO3a transcriptional activity by TSH and insulin

Next, we addressed the question, whether the observed shift in FOXO3a localization after growth factor stimulation correlates with alteration in FOXO3a target gene transcription. The mRNA expression of p27kip and Bim was significantly inhibited after treatment with insulin, serum, or growth media compared with the starving control (for all $P < 0.01$). By contrast, treatment of cells with TSH did not significantly alter mRNA expression of p27kip and Bim compared with starved controls (Fig. 1C).

Furthermore, starving media and TSH appeared to be correlated with the inhibition of FOXO3a-dependent GADD45A transcription, while insulin, serum, and growth media significantly induced GADD45A mRNA expression ($P < 0.001$; Fig. 1C).

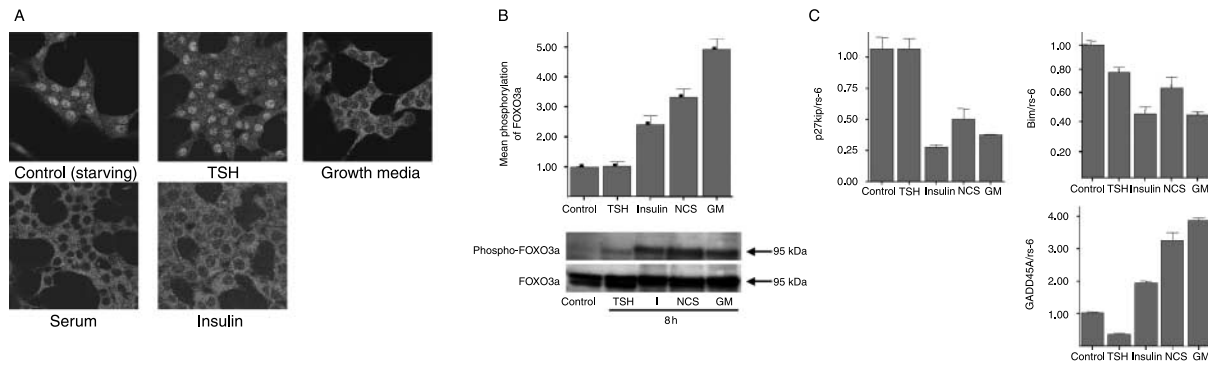


Figure 1 Regulation of FOXO3a localization and function by different growth factors. (A) Confocal microscopy shows that control cells cultured under starving conditions exhibit nuclear accumulation of FOXO3a, which could not be altered by TSH treatment. Conversely, insulin, serum, and full growth media induced nuclear exclusion of FOXO3a and its accumulation in the cytoplasm. (B) Histograms representing the extent of FOXO3a phosphorylation in response to growth factor stimulation. Starving conditions induced a decrease in FOXO3a phosphorylation, whereas addition of insulin, serum, or full growth media increases FOXO3a phosphorylation. TSH stimulation did not significantly alter FOXO3a phosphorylation and localization compared with starved cells. (C) Incubation with serum, insulin, and growth media induced a reduction in p27kip and Bim mRNA expression compared with TSH stimulation and starvation ($P < 0.01$). By contrast, insulin, 5% serum, and full growth media significantly stimulated *GADD45A* mRNA expression compared with TSH incubation and starvation ($P < 0.001$). FRTL5 cells were starved for 48 h (0.2% serum, without TSH and insulin; control) prior to incubation with either 10 nM TSH, 100 ng insulin, 5% newborn calf serum (NCS), full growth media containing insulin, NCS, and TSH (GM), or new starving media (Control) for 8 h. Experiments were performed in duplicate and repeated independently three times. Gene expression was normalized to *rs6* mRNA expression and quantified as described in Materials and Methods section.

FOXO3a expression and activity is differentially regulated by PI3K versus PKA and PKC

To confirm the observed distinct regulation of FOXO3a activity by TSH and insulin, FRTL-5 cells were grown in full growth media, which resulted in an exclusive cytoplasmic FOXO3a expression and FOXO3a Thr32 phosphorylation (Fig. 2A). Subsequently, the cells were stimulated with activators or inhibitors of the cAMP, PI3K/Akt, and PKC signaling pathways. First, induction of the adenylate cyclase by 5 μ M forskolin for 4 h led to a nuclear shift of FOXO3a (Fig. 2A), but this did not result in altered target gene expression. Second, neither the stimulation of PKC with PMA nor its inhibition with GF10923X had an effect on FOXO3a localization (data not shown). Third, inhibition of the PI3K/Akt signaling pathway by LY294002 reversed the effect of insulin on FOXO inactivation and resulted in a nuclear FOXO3a accumulation, and decreased the fraction of FOXO3a phosphorylation (Fig. 2A) and a significant increase in the mRNA expression of the FOXO3a target genes, p27kip ($P < 0.01$) and Bim ($P < 0.01$), compared with the untreated control (Fig. 2B). By contrast, *GADD45A* mRNA expression was significantly downregulated ($P < 0.05$; Fig. 2B).

Oxidative stress results in increased JNK phosphorylation and FOXO3a activation

Thyocytes are frequently exposed to free radicals that are a side product of TH synthesis with hydrogen peroxide required as an essential cofactor. To analyze

FOXO3a activation upon oxidative stress in the thyroid, FRTL-5 cells were treated with 400 μ M H_2O_2 for different periods of time. Control cells were kept in full growth media and showed an exclusive cytoplasmic FOXO3a expression, which correlated with strong FOXO3a (Thr32) phosphorylation (Fig. 3A and B). Prolonged exposure of FRTL-5 cells with 400 μ M H_2O_2 resulted in a nuclear accumulation of FOXO3a and a significant decrease in phosphorylated FOXO3a (Thr32) in the cytoplasm as shown by confocal microscopy (Fig. 3A and B).

In addition, subcellular fractionation was performed and confirmed the decrease in phosphorylated FOXO3a exclusively in the cytoplasm with a combined reduction in total cytoplasmic FOXO3a after H_2O_2 treatment (Fig. 3C). As expected, phosphorylated FOXO3a was absent in the nuclear fraction of controls kept in the growth media (Fig. 3C). Moreover, as a functional readout of FOXO3a activation by oxidative stress, we found a time-dependent increase in the mRNA expression of FOXO3a target genes p27kip, followed by an increase in *GADD45A* transcription after treatment of FRTL-5 cells with 400 μ M H_2O_2 (Fig. 3D).

Analysis of JNK, which has been described as a FOXO signaling partner in other cell systems, showed that increasing H_2O_2 concentrations (400 and 800 μ M for 240 min) led to increased JNK phosphorylation in parallel with the above-described alterations in FOXO localization and activity (Fig. 3B).

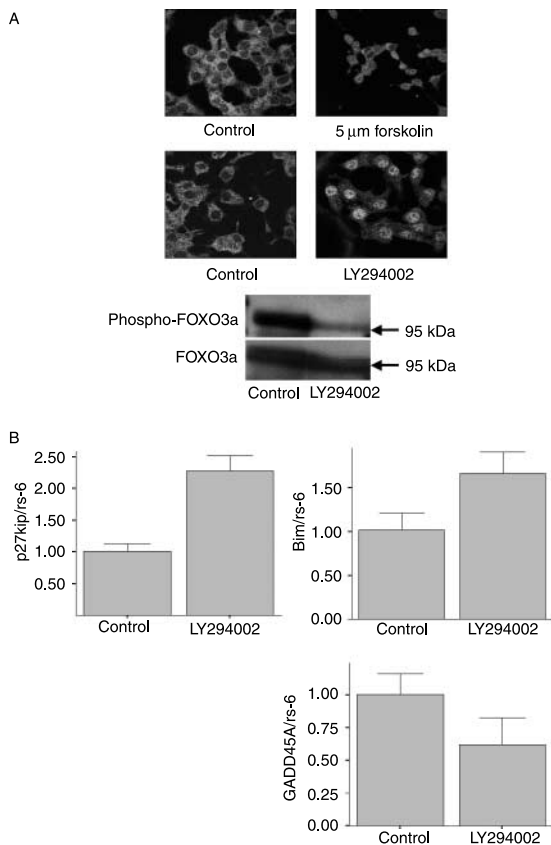


Figure 2 FOXO3a expression and activity is differentially regulated by PKA and PI3K. (A) Treatment of FRTL-5 cells with 5 μ M forskolin for 24 h led to a nuclear shift of FOXO3a, but this did not result in target gene transcription (data not shown), most likely due to a toxic effect of massive cAMP stimulation through forskolin + TSH. A nuclear FOXO3a shift was also observed after inhibition of PI3K by 10 μ M LY294002 and was accompanied by decreased phosphorylation of FOXO3a and (B) increased transcription of the FOXO3a target genes p27kip and *Bim*, but repression of *GADD45A* (for all $P < 0.02$). FRTL5 cells were grown in full growth medium (containing insulin, NCS, and TSH; control) and were incubated with activators of PKA (forskolin) and inhibitors of PI3K (LY294002) for 8 h. Experiments were performed in duplicate and repeated independently three times. Gene expression was normalized to rs6 mRNA expression and quantified as described in the Materials and Methods section.

Expression and transcriptional activity of FOXO3a in thyroid pathologies

Distinct expression and localization of FOXO3a in normal thyroid, follicular adenoma, and thyroid carcinoma

In order to assess the mRNA expression of FOXO3a, we performed quantitative real-time PCR on a panel of NT, FA, and (FTC, PTC). The mRNA of FOXO3a was significantly downregulated in PTC ($P < 0.001$) compared with benign thyroid tumors and normal thyroid tissue (Fig. 4A). In FTC, FOXO3a mRNA

levels were also decreased compared with benign thyroid tumors and NT, but this failed to reach statistical significance ($P < 0.08$).

Subsequently, we investigated FOXO3a protein expression and subcellular localization by immunohistochemistry (Fig. 4B). As demonstrated in Fig. 4C, there is no difference in FOXO3a expression and localization between FA and NT presenting abundant nuclear FOXO3a expression. By contrast, in differentiated thyroid carcinoma (FTC and PTC), a marked cytoplasmatic FOXO3a localization was found, either in addition to nuclear staining (PTC) or with significantly reduced nuclear staining (FTC; Fig. 4B and C).

Correlation between pAkt expression and cytoplasmatic localization of FOXO3a in thyroid carcinoma

Recently, deregulation of the PI3K/Akt pathway has emerged to play a fundamental role in thyroid carcinogenesis (Paes & Ringel 2008). Since we observed an inhibitory impact of the PI3K/Akt pathway on FOXO3a activity *in vitro*, we were interested to know whether this regulatory principle might also apply *in vivo*. Therefore, we performed immunohistochemistry on the same panel of samples already analyzed for FOXO3a protein expression. Thyroid cancers displayed a strong pAkt staining intensity in 65% of the investigated FTC and in 70% of PTC. With the exception of two FTC, all thyroid carcinoma with cytoplasmatic FOXO3a expression showed simultaneously increased Akt phosphorylation. By contrast, pAkt expression was very faint and present in less than 13% of FA and less than 5% of normal thyroids, and this was correlated with an exclusive nuclear FOXO3a localization in these tissues.

Figure 4B shows a representative image of FOXO3a and pAkt expression in normal thyroid tissue, follicular adenoma, and follicular and papillary thyroid cancer.

Differential regulation of FOXO3a target genes in thyroid carcinoma

FOXO3a activity is reflected by target gene transcription, e.g., of *Bim*, *GADD45A*, and p27kip. To investigate whether the different expression and the distinct localization of FOXO3a in normal thyroids, benign, and malignant thyroid pathologies correlates with the mRNA expression of FOXO3a-related target genes, we analyzed the mRNA expression of *GADD45A*, *Bim*, and p27kip in our series of 68 thyroid specimen.

The mRNA expression of p27kip was significantly reduced in thyroid carcinoma compared with benign thyroid tumors and normal thyroid tissue ($P < 0.0001$;

Fig. 5A). In line with this observation, Bim expression was also significantly decreased in malignant thyroid tumors compared with benign thyroid tumors and normal thyroid tissue ($P < 0.02$, data not shown). By contrast, *GADD45A* was significantly upregulated in all thyroid cancers with the highest levels found in FTC ($P < 0.0001$; Fig. 5B).

Discussion

In this work, we investigated for the first time the regulation of FOXO3a in thyrocytes, in particular the influence of growth factors and hydrogen peroxide on

FOXO3a activation and function. In addition, we tried to elucidate the role of FOXO3a in thyroid tumorigenesis.

First, *in vitro*, we demonstrate that FOXO3a expression and activity is differentially regulated by the growth factors TSH and insulin: serum deprivation and stimulation with TSH led to decreased FOXO3a phosphorylation and to nuclear accumulation of FOXO3a, together with an increase in p27kip and Bim and a decrease in *GADD45A* mRNA expression. However, the effect of TSH on FOXO3a activity could only be observed on previously starved cells and could neither be mimicked by endogenous PKA activation with forskolin nor PKC activation with PMA. Hence, the data suggest that TSH has only a minor effect on FOXO3a activity.

By contrast, insulin induced opposing effects with an increased FOXO3a phosphorylation and cytoplasmatic accumulation in line with decreased transcription of Bim and p27kip and increased expression of *GADD45A*. This effect was confirmed by the inhibition of the PI3K/Akt pathway, which resulted in the upregulation of Bim and p27kip mRNA expression. These findings suggest insulin as a negative regulator of FOXO3a activity. However, in line with activation by insulin, abrogation of insulin signaling by LY294002 declined *GADD45A* mRNA expression. Thus, the apparent divergent responsiveness of *GADD45A* compared with p27kip and Bim upon insulin treatment suggests that *GADD45A* transcription directly depends on PI3K activation rather than on FOXO3a activation.

We next turned to the *in vivo* situation in order to study whether FOXO3a expression and function was

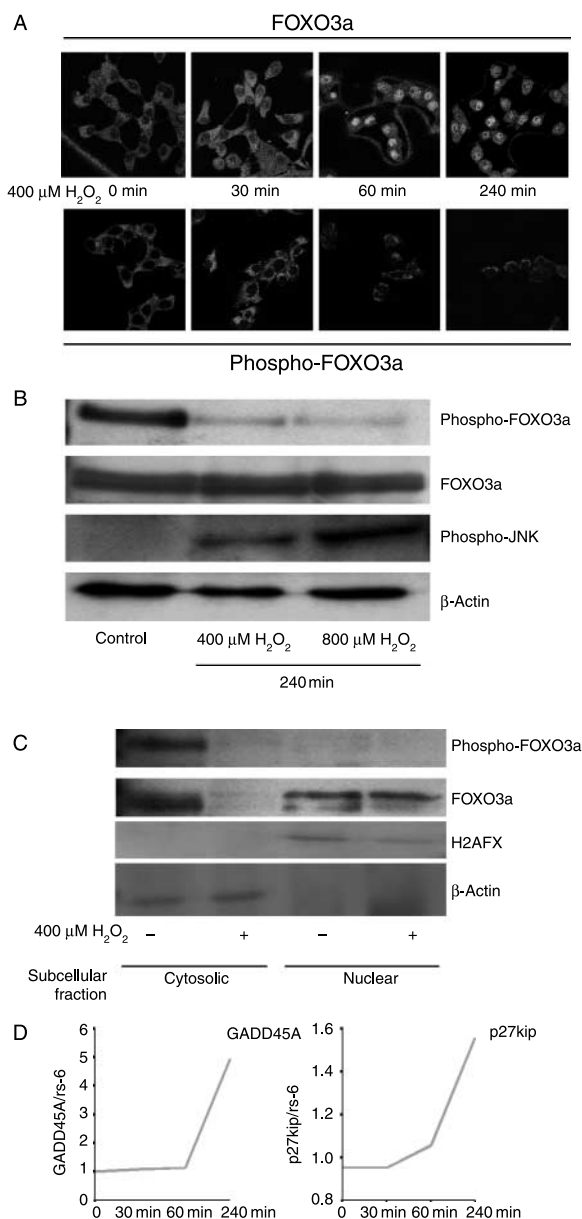


Figure 3 Activation of FOXO3a by oxidative stress.

(A) Confocal microscopy using an anti-FOXO3a/anti-phospho-FOXO3a antibody showed nuclear translocation of FOXO3a in response to prolonged H_2O_2 treatment of FRTL-5 cells with a reduction in phosphorylation of FOXO3a in these cells.

(B) Western blot analysis demonstrating that increasing concentrations of H_2O_2 induce JNK phosphorylation and FOXO3a dephosphorylation on Akt-sensitive phosphorylation sites in FRTL-5 cells. (C) Subcellular fractionation shows a marked decrease in cytoplasmatic phosphorylated FOXO3a and a reduction in cytoplasmatic FOXO3a after treatment of FRTL-5 cells with 400 μM H_2O_2 . As expected, phosphorylated FOXO3a was not detectable in the nuclear fraction of cells. To ensure the separation between the cytosolic and nuclear fraction antibodies against β -actin (cytosol) and H2AFX (nucleus) were used.

(D) Prolonged H_2O_2 treatment of FRTL-5 cells > 60 min led to a time-dependent induction of first p27kip, followed by *GADD45A* expression. FRTL5 cells were grown in full growth medium (containing insulin, serum, and TSH) and were incubated with H_2O_2 in a dose- and time-dependent manner. Experiments were performed in duplicate and repeated independently three times. Gene expression was normalized to rs6 mRNA expression and quantified as described in the Materials and Methods section.

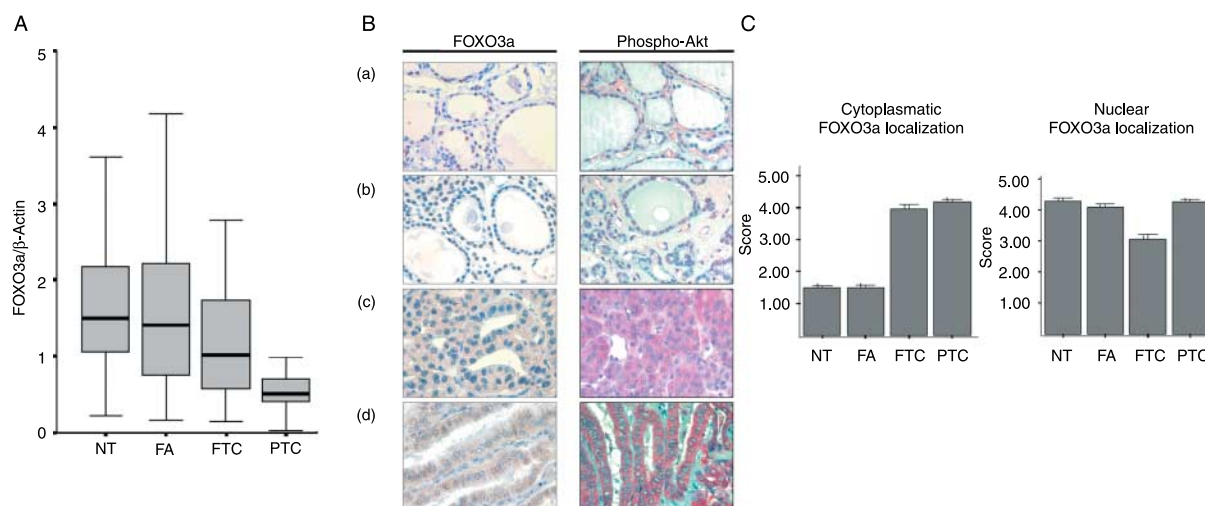


Figure 4 Analysis of FOXO3a and PI3K/Akt in thyroid tumors. (A) The mRNA expression of *FOXO3a* was analyzed in a panel of 22 normal thyroids (NT), 22 follicular adenomas (FA), 14 follicular thyroid carcinoma (FTC), and 10 papillary thyroid carcinoma (PTC). Results are presented in boxplots showing median and distribution (box area = 50% of samples) of *FOXO3a* mRNA expression normalized for β -actin (housekeeping gene). *FOXO3a* mRNA was significantly downregulated in PTC ($P < 0.001$) compared with benign thyroid tumors and normal thyroid tissue. In FTC, *FOXO3a* mRNA levels were also decreased compared with benign thyroid tumors and NT, but this failed to reach statistical significance ($P < 0.08$). (B) Immunohistochemical analysis using antibodies against FOXO3a and pAkt was performed in a series of 196 benign and malignant thyroid tumors including 65 follicular adenoma (FA), 68 follicular thyroid carcinoma (FTC), 63 papillary thyroid carcinoma (PTC) with adjacent normal tissue, and 19 normal thyroid tissues (NT). Representative images of FOXO3a and pAkt staining in (a) NT, (b) FA, (c) FTC, (d) PTC showing nuclear staining in NT and FA and strong cytoplasmatic staining either in addition to nuclear staining in PTC or with significantly reduced nuclear staining in FTC. Phospho-Akt: both thyroid cancers display a strong pAkt-staining intensity, while pAkt staining was very faint/absent in FA and in NT (magnification 400 \times). (C) Histograms presenting the cytosolic and nuclear distribution of FOXO3a in 65 follicular adenoma (FA), 68 follicular thyroid carcinoma (FTC), 63 papillary thyroid carcinoma (PTC) with adjacent normal tissue, and 19 normal thyroid tissues (NT). Semiquantitative analysis was performed as described in the Materials and Methods section (0 = 0% staining, 1 \geq 25% staining, 2 = 25–30% staining, 3 = 50–75% staining, 4 \geq 75% staining).

altered between benign and malignant thyroid tumors and normal thyroid tissue. We observed two aspects both pointing to impaired FOXO function: first, a downregulation of *FOXO3a* mRNA was found in thyroid cancers, reaching significance for PTC. Secondly, and most interestingly, we found a striking difference in the subcellular localization of FOXO3a in thyroid carcinoma compared with FA and normal thyroids: in differentiated thyroid carcinoma (FTC and PTC), a cytoplasmatic FOXO3a localization was found, either in addition to nuclear staining (PTC) or with significantly reduced nuclear staining (FTC). There was no difference in FOXO3a expression and localization between follicular adenoma and normal thyroid tissue. These data also fit well with the decreased mRNA expression of the FOXO3a target genes p27kip and Bim and increased *GADD45A* levels detected in the thyroid malignancies.

Several aspects emerging from our observations involve the insulin/PI3K/Akt pathway as an inhibitory switch of FOXO3a function. The finding of the increased expression of *GADD45A* in FTC and PTC despite the nuclear absence of FOXO3a correlates

perfectly with the observed dependence of *GADD45A* on insulin/PI3K/Akt signaling. There is emerging evidence that the PI3K/Akt axis is constitutively active in many types of human cancer (Nicholson & Anderson 2002). A deregulation of the PI3K/Akt activation can occur due to mutations or amplification of *PI3K/Akt* genes or as a result of decreased activity or loss of PTEN (Nicholson & Anderson 2002). For instance, in thyroid cancer, it has been shown that the insulin/PI3K/Akt axis is frequently constitutively activated, e.g., by *PI3KCA* mutations and *PI3KCA* copy gains, as well as PTEN mutations in FTC (Dahia et al. 1997, Paes & Ringel 2008). Moreover, PI3K/Akt signaling also appears to be involved in the progression of both papillary and follicular cancers to anaplastic thyroid cancers (Shino-hara et al. 2007). A constitutive activation of the insulin/IGF1/PI3K/Akt axis might explain the observed cytoplasmatic accumulation of FOXO3a leading to the inhibition of its transcriptional function, as demonstrated by the downregulation of p27kip and Bim in the thyroid carcinomas and *in vitro* after insulin stimulation. Furthermore, high levels of pAkt expression in FTCs and PTCs correlated with increased cytoplasmatic

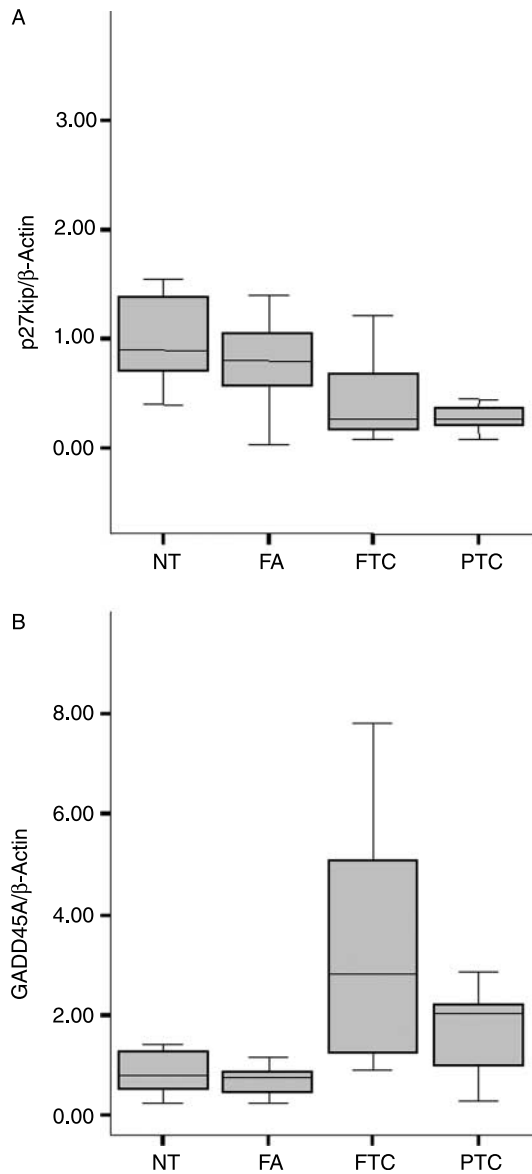


Figure 5 Analysis of mRNA expression of FOXO3a target genes in benign and malignant thyroid tumors. The mRNA expression of the FOXO3a target genes p27kip and *GADD45A* was analyzed in a panel of 22 normal thyroids (NT), 22 follicular adenoma (FA), 14 follicular thyroid carcinoma (FTC), and 10 papillary thyroid carcinoma (PTC). Results are presented in boxplots showing median and distribution (box area = 50% of samples) of FOXO3a mRNA expression normalized for β-actin (housekeeping gene). (A) p27kip mRNA expression is significantly downregulated in FTC and PTC compared with FA, NT; $P < 0.001$. (B) *GADD45A* mRNA expression is significantly upregulated in FTC and PTC compared with NT and FA ($P < 0.0001$).

localization of FOXO3a in FTCs and PTCs. The pathophysiological consequences, however, at this state can only be speculative: our findings may suggest evidence for a novel escape mechanism from

p27kip-induced cell cycle arrest and Bim-promoted apoptosis in thyroid cancer cells through pAkt-mediated inactivation of FOXO3a, thereby driving cancer cells from the inhibition of growth to cellular proliferation. A failure to induce cell cycle arrest at the G1/S boundary has already been shown in p27-deficient fibroblasts (Shinohara *et al.* 2007). In addition, it has been shown that Bim^{-/-} lymphocytes are more resistant to cell death after cytokine withdrawal (Bouillet *et al.* 1999).

The observed upregulation of the growth arrest and DNA damage-inducible gene *GADD45A* in thyroid carcinomas is more difficult to explain since the function of *GADD45A* has been linked to growth control by induction of cell cycle arrest at the G2/M boundary, apoptosis, and DNA repair (Hollander *et al.* 1999, Wang *et al.* 1999, Tong *et al.* 2001). Several explanations for the observed *GADD45A* upregulation in thyroid carcinomas may have to be considered. First, *GADD45A* may be upregulated as a compensatory mechanism, which is yet ineffective due to other factors overriding cell cycle arrest (such as p27kip and Bim downregulation). Second, *GADD45A* may be non-functional due to mutations that have been reported for several types of human cancer (Yamasawa *et al.* 2002, Zerbini & Libermann 2005). Third, instead of an exclusive tumor-suppressive role, overexpression of *GADD45A* may counterbalance DNA damage-mediated apoptosis in cancer cells. For instance, *in vitro* models using bone marrow cells from *GADD45A*-deficient mice identified *GADD45A* as an anti-apoptotic gene, which increases the survival of hematopoietic cells upon u.v. radiation (Gupta *et al.* 2006). Fourth, *GADD45A* is also positively regulated by the PI3K/Akt signaling cascade and this appears to be independent of FOXO3a. Thus, we could demonstrate in FRTL-5 cells that *GADD45A* transcription is induced by the PI3K/Akt signaling cascade in response to insulin stimulation, whereas inhibition of the PI3K/Akt pathway by Ly294002 significantly reduced the expression of *GADD45A* mRNA. This dichotomous principle of *GADD45A* regulation has, to our knowledge, not hitherto been reported and will be the subject of future studies.

In conclusion, the data presented herein provide strong evidence for a novel mode of the insulin/IGF1 pathway in the regulation of thyroid cell proliferation and apoptosis by controlling sequestering of FOXO3a into the cytoplasm through phosphorylation. This effect is particularly apparent in FTCs and may be linked to constitutive activation of the PI3K/Akt pathway in these tumors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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