Direct control of caveolin-1 expression by FOXO transcription factors

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Protein kinase B can phosphorylate and thereby inactivate the FOXO (forkhead box O) family of transcription factors. When active, FOXO factors can bind to DNA in promoter sequences and subsequently regulate gene expression. We have used DNA microarray analysis to identify potential gene targets of FOXO. In the present study we demonstrate that caveolin-1 is directly controlled by FOXO. Firstly, caveolin-1 expression was increased upon induction or over-expression of FOXO factors at both mRNA and protein levels. Second, we show that endogenous regulation of FOXO activity regulates caveolin-1 levels and that this can be inhibited by dominant-negative FOXO. Third, FOXO activates transcription from the caveolin-1 promoter, and using chromatin

immunoprecipitations we demonstrated that this activation occurs via direct interaction of FOXO with the promoter. Finally, we demonstrate FOXO-mediated attenuation of EGF (epidermal growth factor)-induced signalling, which in part is mediated by caveolin-1 expression, as suggested by previous studies [Park, Park, Cho, Kim, Ko, Seo and Park (2000) *J. Biol. Chem.* **275**, 20847–20852]. These findings suggest a novel mechanism by which FOXO factors can exert their cellular effects via transcriptional activation of caveolin-1.

Key words: FOXO, caveolin-1, insulin, transcription, protein kinase B/Akt.

INTRODUCTION

FOXO (forkhead box O) transcription factors are the human homologues of the nematode *Caenorhabditis elegans* transcription factor DAF-16 (decay accelerating factor 16). Both FOXO and DAF-16 are winged-helix-domain-containing proteins that are under the direct control of the insulin–PI3K (phosphoinositide 3-kinase)–PKB (protein kinase B) signalling cascade. FOXOs and DAF-16 are phosphorylated by PKB within conserved consensus phosphorylation motifs, leading to nuclear exclusion and subsequent transcriptional inactivation. In addition to PKB-mediated phosphorylation, it is now clear that transcriptional activity can also be regulated by phosphorylation by other kinases and by other post-translational modifications, such as acetylation ([1–3] and reviewed in [4]). A steadily increasing amount of publications places this pathway, and in particular the FOXO proteins, in various cellular processes such as apoptosis, differentiation and cell cycle arrest, and via these processes in phenomena such as aging (reviewed in [4]). FOXO proteins can bind promoter regions of target genes that contain consensus binding elements (5'-TTGTTTAC-3') and thereby regulate their expression [5]. Several target genes have been identified thus far. By inducing gene transcription of pro-apoptotic genes such as Bim and FasL, but also others such as Bcl6, FOXOs can trigger haematopoietic cells to go into apoptosis [6–8]. In other cell types FOXO activation leads to a G₁ cell cycle arrest via regulation of p27^{kip1} and cyclin D, or a G₂ arrest via GADD45 (growth-arrest and DNA-damage-inducible protein 45) regulation [9–11]. By regulating levels of proteins in the insulin pathway, such as the insulin receptor, it can also create a feedback loop [12].

Caveolin-1 is the main constituent of microdomains localized within the cellular membrane called caveolae. Within caveolae, caveolin-1 interacts with growth factor receptors, such as EGF (epidermal growth factor) and insulin receptors, and other signal-

ling molecules, such as PKA (protein kinase A), Src kinases and H-Ras [13,14]. Through this interaction these proteins are mostly negatively regulated with respect to their activity. The human caveolin-1 gene is situated on gene locus 7q31.1, and through alternative transcription initiation sites, transcription results in α and β isoforms of 24 and 22 kDa respectively. This gene locus has been implicated in tumorigenesis, but it is still controversial how caveolin-1 plays a role in this process. Nevertheless, caveolin-1^{-/-} mice do show hyperproliferative abnormalities and acceleration of mammary lesion formation in tumour-prone transgenic mice [15,16]. Thus a decrease in caveolin-1 expression may contribute to increased cell proliferation and thereby to tumorigenesis. Consistent with this idea, a decrease in caveolin-1 expression leads to an exit from cell cycle arrest, whereas overexpression of caveolin-1, induced by hydrogen peroxide, leads to G₀/G₁ cell cycle arrest and premature cellular senescence [17,18]. Caveolin-1 expression in senescent cells is elevated and leads to attenuation of EGF signalling with respect to MAPK (mitogen-activated protein kinase) phosphorylation [19]. This senescent phenotype, however, can be reversed by reducing the expression levels of caveolin-1 [20], suggesting that either the senescent state is a reversible phenotype or that these cells are actually quiescent, rather than senescent.

The molecular mechanisms regulating caveolin-1 expression are largely unknown. Various signalling pathways have been implicated, but a coherent picture is still lacking. It is known that forskolin via cAMP can down-regulate mRNA levels in a dose-dependent manner [21]. Also c-Myc can directly regulate expression via the INR (insulin receptor) sequence present in its promoter [22]. PKC ϵ (protein kinase C ϵ) signalling can enhance caveolin-1 expression as well [23]. Certain physiological processes are also able to regulate caveolin-1 expression like lactation, via prolactin–Ras-dependent signalling, and adipogenesis/differentiation [24,25]. The latter process can include a

Abbreviations used: ChIP, chromatin immunoprecipitation; DAF-16, decay accelerating factor 16; EGF, epidermal growth factor; FoxO, forkhead box O; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; 4OHT, 4-hydroxytamoxifen; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; Rb, retinoblastoma; siRNA, small interfering RNA; TK, thymidine kinase; TKO, triple knock-out.

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role for PPAR γ (peroxisome-proliferator-activated receptor γ), since it can up-regulate caveolin-1 expression and can lead to adipogenesis [26]. Furthermore caveolin-1 is down-regulated in the S-phase of dividing cells and up-regulated by free cholesterol via two sterol-regulatory-element-like sequences [27,28]. It is suggested that this occurs via E2F and p53 [29].

To identify novel target genes of FOXO transcription factors, DNA microarray analysis was performed using a human colon carcinoma cell line stably expressing an inducible active FOXO3a. One of the genes that was found to be up-regulated upon FOXO3a activation was caveolin-1. In the present study, we show that caveolin-1 expression is directly regulated by FOXO and transcriptional activation of the caveolin-1 promoter by FOXO leads in part to attenuation of EGF-induced signalling.

EXPERIMENTAL

Constructs

The retroviral construct pBabe-DB expressing the N-terminally HA (haemagglutinin)-tagged DNA binding region of FOXO4 was used as a dominant-negative FOXO and was constructed by subcloning from pMT2-HA-DB [9]. The *PstI/XbaI*-digested fragment was blunted and ligated into blunted *EcoRI*-digested pBabe-puro vector. pBabe-puro was used as negative control in retroviral infections. The luciferase expression construct containing the caveolin-1 promoter fragment encompassing the putative FOXO binding site (pGL2-cav1) was constructed as follows. The caveolin promoter region from -2080 to -1569 was amplified by PCR and cloned into pGEM-T (Promega) using PCR primers with flanking *SacI/XhoI* sites. Primers used were 5'-GAGCTCGCTGCAGTGACCTATGAATG-3' and 5'-GTCGACGAACTCATGGAAACAAATAGGG-3'. Subsequently the *SacI/XhoI* fragment was subcloned into *SacI/XhoI*-digested pGL2-basic (Promega). The following constructs have been described previously: retroviral N-terminally HA-tagged FOXO3a, in which the PKB phosphorylation motifs have been mutated (pBabe-HA-FOXO3a-A3) [30], mammalian expression vector for N-terminally HA-tagged wild-type FOXO4 (pMT2-HA-FOXO4) [9]. The retroviral construct pBabe-p16, expressing the cell cycle inhibitor p16^{Ink4A}, was a gift from Professor Dr Rene Medema (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands) [31]. The control luciferase construct pRL-TK (thymidine kinase *Renilla* luciferase) was purchased from Promega. The following siRNA (small interfering RNA) oligonucleotides (Dharmacon) directed against caveolin-1 were used: 5'-TGTGATTGCAGAACAGAA-3' and 5'-GCCGTGTCTATTCCATCTA-3'.

Cell culture and transient transfection

DLD1 and DL23 cells were grown in RPMI 1640 medium. HEK-293T, C2C12, A14 and the MEFs (mouse embryonic fibroblasts) [wild-type and TKO (triple knock-out)] were grown in DMEM (Dulbecco's modified Eagle's medium) and Mcf7 cells in DF12 medium. Media were supplemented with 10% fetal calf serum (Bio-Whittaker), 1% penicillin/streptomycin (Bio-Whittaker) and 2 mM L-glutamine (Bio-Whittaker). DLD1 and DL23 cells were treated with 0.5 μ M 4OHT (4-hydroxytamoxifen) where indicated. EGF was added to a final concentration of 20 ng/ml. Cells were treated overnight with 1 μ M insulin or 10 μ M LY294002 in medium without serum. TKO MEF cells were a gift from Dr Hein te Riele (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands) [32]. A14 cells were transiently transfected using the CaPO₄ method,

and HEK-293T cells using PEI (Polysciences). Transfection of siRNA oligonucleotides was performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol, and after 48 h the cells were harvested.

Retroviral infection

Retroviruses were obtained by transfecting Phoenix cells with the relevant retroviral construct. After transfection (36 h) conditioned medium from these cells was collected, filtered and diluted 1:1 with fresh medium, and 6 μ g/ml polybrene (Sigma) was added. The medium was added in two consecutive rounds to infect MEFs, and infected cells were selected for by puromycin treatment and collected after 48 h.

Northern blotting

Total RNA was extracted from cells using RNazol (Tel-Test, Friendswood, TX, U.S.A.). RNA (20 μ g) was subjected to electrophoresis, blotted on to nitrocellulose and probed for caveolin-1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using [α -³²P]dCTP-labelled cDNA probes.

Immunoblot analysis

Protein samples in Laemmli buffer were separated by SDS/PAGE on 12.5% gels and transferred on to PVDF membranes (NEN). Western blots were blocked overnight at 4°C in TBS (Tris-buffered saline)/0.1% Tween-20 (TBST) containing either 2% non-fat dried milk (Protifar, Nutricia) and 0.5% BSA (Sigma), or 2% BSA for the anti-phosphorylation-specific antibodies. The Western blots were then incubated for 2 h with the indicated primary antibodies in TBST using the dilutions recommended by the manufacturers or 1:8000 for the self-generated antibodies. After washing four times for 5 min with TBST blots were incubated with secondary antibodies anti-mouse HRP (horseradish peroxidase) or anti-rabbit HRP (1:10000) for 1 h at 4°C. Blots were washing again four times for 5 min with PBS/0.1% Tween-20 and analysed using chemiluminescence (NEN). Primary antibodies used were anti-caveolin-1 against the α isoform (N-20; Santa Cruz) and anti-caveolin-1 against both α and β isoforms (clone 2297; BD Transduction Laboratories), anti-p27^{kip1} (BD Transduction Laboratories), anti-phospho-p44/42 MAPK (phosphorylation at Thr²⁰²/Tyr²⁰⁴) and anti-phospho-PKB (Ser⁴⁷³) (Cell Signaling), anti-tubulin (Calbiochem), anti-HA (12CA5) and anti-PKB (5178) [33].

ChIP (chromatin immunoprecipitation)

DL23 cells were incubated with or without 4OHT for 20 h before ChIP analysis was performed. This was performed as described previously [34], with some modifications. Lysates were sonicated 4 times for 30 s on ice at 40% using an UP200S sonicator (Dr Hielscher, GmbH, Teltow, Germany). After spinning down, 3% of the supernatant was taken as input sample after which the remainder was divided in two equal amounts that were diluted 5-fold in dilution buffer (20 mM Tris, pH 8.0, 1% SDS, 2 mM EDTA, 0.3 mM NaCl, herring sperm) and pre-cleared two times using Protein A beads. For the immunoprecipitation the anti-HA antibody (12CA5) was used and anti-PKB antibody (5178) as negative control. After the 16-h incubation period with the primary antibody, Protein A beads were added and incubated for another 45 min, followed by the washing and elution steps described in [34]. RNase and NaCl were added (to a final concentration of 0.1 μ g/ μ l and 300 mM respectively) and heated for 16 h at 65°C to dissociate the cross-linked portion. After precipitation, proteinase K treatment and phenol/chloroform

extraction, DNA was analysed by PCR (50 ng of each primer set, 2 units *Taq* polymerase; at 60 °C, 29 cycles). Primer sets used were 5'-GCTGCAGTGACCTATGAATG-3' and 5'-GAACTCATGG-AAACAAATAGGG-3' for the caveolin-1 PCR, resulting in a 511 nt fragment encompassing the FOXO binding element, and 5'-GAATATCCGATCTAGCCTGG-3' and 5'-TGGCACTGTGCTTCCTGTAC-3' for the control PCR, resulting in a 370 nt fragment.

Luciferase assay

Cells were transfected with 1 µg of pGL2-cav1 along with 20 ng of TK-*Renilla* and various concentrations of HA-FOXO4 (0.5 and 2.0 µg for A14 cells and 0.2, 0.5 and 1.0 µg for HEK-293T cells). Lysis and subsequent determination of luciferase counts were performed 40 h after transfection in triplicate using the Dual-Luciferase Reporter Assay (Promega). The assays were performed four times.

RESULTS

Caveolin-1 expression is increased by FOXO activity

To identify novel transcriptional targets of FOXO transcription factors a microarray was performed using the DL23 cell line. This subclone from the DLD1 human colon carcinoma cell line expresses the conditionally active HA-FOXO3a-A3-ER construct [35]. This FOXO construct is N-terminally HA-tagged to allow detection using the 12CA5 monoclonal antibody, it has been mutated within its three PKB phosphorylation sites so that it can no longer be inhibited by PKB-mediated phosphorylation, and it is tagged C-terminally to a modified form of the oestrogen receptor hormone-binding domain so that HA-FOXO3a-A3 is inactive unless cells are treated with 4-OHT. A putative target gene that was found to be up-regulated upon 4-OHT treatment of DL23 cells in the gene expression microarray was caveolin-1. Transcriptional regulation of caveolin-1 has been studied previously and the promoter region has been determined [27]. To analyse the putative FOXO-mediated transcriptional regulation we examined the region upstream of the transcription initiation site of the human caveolin-1 gene using the GenomeView program [NCBI (National Center for Biotechnology Information) Entrez Genome]. This revealed one consensus FOXO binding sequence (5'-TTGTTTAC-3') at position -1814 upstream of the transcription initiation site and three nearly perfect sequences on positions -1584, -1069 and -679.

To verify the results from the microarray, we analysed caveolin-1 expression following FOXO activation. Induction of FOXO activity by 4-OHT treatment in DL23 cells resulted in a time-dependent increase of caveolin-1 RNA and protein levels (Figures 1A and 1B respectively). Using a different anti-caveolin-1 antibody recognizing both α and β isoforms of caveolin-1, we showed that the induction of caveolin-1 transcripts leads to an increase of both isoforms (Figure 1B). Having verified our DNA microarray result we next wanted to know whether FOXO would also induce caveolin-1 expression in an unrelated experimental system. To this end, MEFs were virally infected to express active FOXO3a. Protein analysis showed increase in caveolin-1 expression (Figure 1C). Thus we concluded that ligand-independent FOXO activation results in an increase in caveolin-1 RNA and protein expression.

Regulation of caveolin-1 expression by endogenous signalling

As our results show that over-expression of FOXO can regulate caveolin-1 expression, we next examined whether caveolin-1 expression could also be regulated by endogenous insulin-PI3K-

Figure 1 FOXO activation induces caveolin-1 expression

(A) DL23 and DLD1 cells were treated with 4-OHT for the indicated times. RNA was isolated and Northern blotting was performed for caveolin-1 and GAPDH. (B) DL23 and DLD1 cells were treated with 4-OHT for the indicated durations. Protein samples were collected, equalized for protein content and analysed by immunoblotting for caveolin-1 using two different antibodies: cav-1(SC) recognizing caveolin-1 α and cav-1(TL) recognizing caveolin-1 α and caveolin-1 β . Anti-p27^{kip1} antibody was used as positive control for FOXO3a activation [9] and anti-tubulin antibody to ensure equal loading. (C) Wild-type MEFs were retrovirally infected with pBabe-puro or pBabe-HA-FOXO3a-A3 (FH) and equalized protein samples were analysed for caveolin-1, p27^{kip1}, HA and tubulin levels.

PKB-FOXO pathway signalling. To this end we made use of C2C12 mouse myoblasts and A14 cells, NIH3T3 cells that are stably expressing the human insulin receptor [36]. In both cell lines insulin treatment induces PKB activation, as can be observed by Western blotting using an antibody against phosphorylated PKB (Figure 2B). In both cell lines the RNA and protein levels of caveolin-1 were up-regulated by overnight serum deprivation (results not shown) or treatment with LY294002 (Figures 2A and 2B). On the other hand insulin treatment resulted in a decrease in caveolin-1 expression (Figures 2A and 2B). This indicates that caveolin-1 expression is regulated by endogenous insulin signalling via PI3K. To further demonstrate the involvement of FOXO in endogenous regulation of caveolin-1 expression we virally infected MEFs to express the isolated DNA-binding region of FOXO4 (DB), which acts as a dominant-negative form of FOXO [9]. Cells were then cultured in the presence of fetal calf serum or serum deprived for 24 h. As can be seen in Figure 2(C) dominant-negative FOXO could inhibit caveolin-1 expression in both the serum-containing and serum-deprived conditions. However, quantification of three independent experiments using

Figure 2 Endogenous FOXO activity regulates caveolin-1 expression

(A) A14 and C2C12 cells were treated overnight with LY294002 (LY) or insulin (i) in serum-free medium. RNA samples were collected and analysed using caveolin-1- and GAPDH-specific probes. (B) A14 and C2C12 cells were treated overnight with LY294002 or insulin in serum-free medium. Equalized protein samples were immunoblotted for the presence of caveolin-1 and actin (upper two panels). As control for the regulation of the insulin pathway immunoblotting was performed for phosphorylated PKB (phospho-Ser⁴⁷³) and total PKB (lower two panels). (C) Wild-type MEFs were retrovirally infected with pBabe-puro or pBabe-DB. Cells were subsequently left untreated or serum-starved for 20 h. Protein samples were collected, equalized for protein content and analysed using antibodies against caveolin-1 and the indicated proteins. Quantification of three independent experiments using ImageQuant showed that in the presence of the dominant-negative FOXO the induction of caveolin-1 levels by serum-deprivation was reduced from 2.8 ± 0.4 - to 1.5 ± 0.2 -fold.

ImageQuant showed that in the presence of the dominant-negative FOXO the induction of caveolin-1 levels by serum deprivation was reduced from 2.8 ± 0.4 - to 1.5 ± 0.2 -fold. This reduction occurs despite the small down-regulation of PKB activity in DB-infected cells. From this we conclude that caveolin-1 expression is, at least in part, regulated by endogenous signalling regulating FOXO.

FOXO factors regulate expression of caveolin-1 independent of cell cycle regulation

It has been described that FOXO activation leads to cell cycle arrest and that this occurs via regulation of p27^{kip1} and cyclin D gene transcription [9,10]. For caveolin-1, it has been described that up-regulation of caveolin-1 itself can induce senescence [18]. Senescent cells are cells that are irreversibly arrested in the G₀/G₁ phase of the cell cycle. Consistent with caveolin-1 inducing senescence it has also been described that senescent cells display increased levels of caveolin-1. To gain insight whether the observed increase in caveolin-1 expression following FOXO

activation is due to direct transcriptional control by FOXO, or a consequence of FOXO-induced cell cycle arrest. We analysed caveolin-1 expression in cells arrested by the expression of p16^{ink4a}, an inhibitor of the cell cycle-dependent kinases responsible for G₁ progression [37,38]. Indeed, viral infection of MEFs with p16^{ink4a}, but also FOXO3a-A3 induced a potent cell cycle arrest in G₁ (Figure 3A), and induced an increase in caveolin-1 mRNA levels (Figure 3A). Thus this result shows that caveolin-1 expression unexpectedly may be under the direct control of p16^{ink4a}, or that indeed caveolin-1 expression can increase as a consequence of G₁ cell cycle arrest. We therefore wanted to know whether caveolin-1 regulation by FOXO was via a direct mechanism or merely the result of a FOXO-induced cell cycle arrest. To study this we made use of TKO MEFs that are deficient for all three members of the Rb (retinoblastoma) pocket protein family (Rb, p107 and p130). It has been described that these cells are insensitive to serum deprivation with respect to cell cycle regulation [32]. We confirmed this and also observed no regulation of the cell cycle by insulin in these cells (results not shown). Thus serum-deprived TKO MEFs were either left untreated or overnight stimulated with insulin. Caveolin-1 levels, as well as the levels of the positive control p27^{kip1}, were decreased upon insulin treatment (Figure 3B). From this we conclude that the insulin-PI3K pathway regulates caveolin-1 expression directly and in a cell-cycle-independent manner.

FOXO factors directly regulate expression of caveolin-1

As the results described above clearly indicate direct regulation of caveolin-1 expression by FOXO, we next performed ChIPs to demonstrate that FOXO indeed can bind directly to the caveolin-1 promoter in order to activate transcription of the caveolin-1 gene. Chromatin-bound DNA was isolated from DL23 cells that were left untreated or stimulated with 4-OHT for 20 h. ChIPs were carried out using an anti-HA antibody to pull down HA-tagged FOXO3a-A3, or anti-PKB antibody as a negative control. Figure 3(C) shows that only in the cells stimulated with 4-OHT FOXO3a was able to bind the caveolin-1 promoter. This was visualized by PCR on a caveolin-1 promoter fragment containing the consensus FOXO binding site and subsequent Southern blotting using a labelled caveolin-1 promoter fragment obtained by PCR. A control PCR encompassing a non-specific genomic region nearby the caveolin-1 gene displayed only positive amplification in the input lanes, indicating that the caveolin-1 promoter immunoprecipitation was specific.

Being able to show that FOXO binds to the caveolin-1 promoter we wanted to know whether this binding would indeed lead to transcriptional activation of caveolin-1 gene expression. To this end the PCR product obtained, encompassing the FOXO binding element at -1814, was cloned into a luciferase vector (pGL2-cav1). A14 and HEK-293T cells were transiently transfected with this pGL2-cav1, together with increasing amounts of HA-tagged FOXO4. In both cell lines expression of FOXO4 induces transcriptional activity in a dose-dependent manner (Figure 3D). This shows that FOXO binding to this region can confer transcriptional regulation of caveolin-1 gene expression by FOXO. However, the luciferase reporter used contains a fragment of the caveolin-1 promoter encompassing the one perfect consensus binding element for FOXO, and it is possible that the other three imperfect FOXO elements that are also present within the promoter may play a role in the transcriptional activation by FOXO as well.

FOXO activation attenuates EGF-induced MAPK phosphorylation

It has been reported that caveolin-1 negatively affects EGF signalling by attenuating downstream signalling, i.e. MAPK

Figure 3 FOXO directly regulates caveolin-1 expression

(A) Wild-type MEFs were retrovirally infected with pBabe-puro, pBabe-p16 or pBabe-HA-FOXO3a-A3 (FH). RNA samples were collected and analysed by Northern blotting for caveolin-1 and GAPDH. Cell cycle profiles visualized using propidium iodide are shown together with the percentage of cells in the indicated phases of the cell cycle. (B) TKO MEFs were serum-deprived for 24 h in the absence or presence of insulin. Whole cell lysates were collected, equalized for protein content and analysed for the presence of caveolin-1, p27^{kip1} and tubulin. (C) ChIPs were performed using DL23 cells treated with or without 4-OHT for 20 h. Negative and positive controls were milliQ and genomic DNA from DL23 cells respectively. — H, anti-HA immunoprecipitation performed on nuclear lysis buffer/dilution buffer; 1, input; 2, anti-PKB immunoprecipitation; 3, anti-HA immunoprecipitation. (D) A14 and HEK-293T cells were transiently transfected with cav1-luciferase construct, TK-*Renilla* and increasing amounts of HA-FOXO4. Cells were lysed and luciferase activity was measured. Results were analysed using one-way ANOVA and were highly significant ($P < 0.001$). Expression of HA-FOXO4 was analysed using SDS/PAGE and anti-HA antibody.

phosphorylation and subsequent effects. This desensitization of growth factor signalling is a hallmark of senescence, but may equally apply to quiescence [19]. The FOXO-mediated increase in caveolin-1 expression may therefore result in attenuation of EGF signalling. To study whether FOXO activation would indeed show attenuation of growth-factor-induced signalling we used DL23 cells treated or untreated with 4-OHT. As shown above (Figure 1) cells treated with 4-OHT showed elevated caveolin-1 expression, and phosphorylation of MAPK upon EGF stimulation was inhibited by FOXO activation, whereas no effect of insulin-induced MAPK phosphorylation was observed (Figure 4A). In agreement with previous results [19], this suggests that activation of FOXO attenuates EGF signalling through up-regulating caveolin-1 expression. To show that the increase in caveolin-1 expression by FOXO is responsible for this observed MAPK attenuation after EGF treatment, we inhibited FOXO-induced caveolin-1 expression by using different siRNAs for caveolin-1 (Figure 4B). The FOXO-induced increase in caveolin-1 was inhibited by the different siRNAs, albeit that the efficiency whereby caveolin-1 induction was repressed varied. siRNA against caveolin-1 partially restored EGF-induced MAPK phosphorylation. This implies at least in part a direct role for caveolin-1 expression in the attenuation of MAPK phosphorylation. As EGF-induced MAPK phosphorylation was not fully rescued, we tested whether in a different model system loss of caveolin-1 expression would affect MAPK phosphorylation differently. Therefore, we transfected insulin-responsive A14 cells with siRNA for caveolin-1. After serum deprivation, cells were

stimulated with insulin leading to a modest MAPK phosphorylation, which was slightly enhanced by lowering caveolin-1 expression (Figure 4C). This result again shows that caveolin-1 expression can modulate MAPK phosphorylation after growth factor treatment of cells, but that the effects are modest. In addition to attenuating MAPK phosphorylation, it has also been shown that caveolin-1 expression can lead to PKB activation through sequestering PP2A (protein phosphatase type 2A), the phosphatase for PKB [39]. In our DL23 cell system we stimulated FOXO by addition of 4-OHT, resulting in increased caveolin-1 protein levels and PKB phosphorylation. Introduction of caveolin-1 siRNA again lowered caveolin-1 protein levels and resulted, as with MAPK phosphorylation, in a partial attenuation of PKB phosphorylation (Figure 4D). Thus we concluded that caveolin-1 expression does modulate growth factor-induced signalling, but that in the DL23 cells FOXO is likely to also induce other regulators of MAPK and PKB phosphorylation. For PKB regulation a possible other regulator could be IRS-2 (insulin receptor substrate 2), which was recently described as transcriptionally regulated by FOXO [40].

DISCUSSION

In the present study we show that FOXO over-expression induces caveolin-1 expression and that endogenous regulation of caveolin-1 by growth factor signalling is mediated by FOXO. Furthermore, we demonstrate that this regulation of caveolin-1 gene expression

Figure 4 FOXO activation attenuates EGF-induced MAPK phosphorylation

(A) DL23 cells were treated with or without 4-OHT for 20 h and subsequently stimulated with or without EGF for 10 or 20 min. Whole cell lysates were collected, equalized for protein content and then analysed for phosphorylated MAPK (P-MAPK), phosphorylated PKB (P-PKB), caveolin-1 (cav-1) and tubulin. (B) DL23 cells were transiently transfected with different siRNA constructs directed against caveolin-1. Cells were then treated with or without 4-OHT for 20 h and stimulated with or without EGF for 10 min. Immunoblotting was performed on equalized protein samples for the indicated proteins. (C) A14 cells were transiently transfected with different siRNA constructs directed against caveolin-1. After overnight serum deprivation cells were stimulated with insulin for 10 min. Whole cell lysates were equalized for protein content and analysed for phosphorylated MAPK, caveolin-1 and tubulin. (D) DL23 cells were transiently transfected with two different caveolin-1 siRNA oligonucleotides and subsequently treated with or without 4-OHT for 20 h. Cells were then stimulated with or without EGF for 10 min and equalized protein samples were subjected to immunoblotting for the presence of the indicated proteins.

by FOXO is through direct binding of FOXO to the caveolin-1 promoter region. As we could show in most experiments a similar effect on caveolin-1 of both FOXO3a and FOXO4, this suggests that caveolin-1 is a general target of the FOXO family. This is consistent with most other described FOXO target genes for which no specific involvement of either FOXO is demonstrated. This up-regulation of caveolin-1 protein by FOXO results in part in the apparent down-regulation of EGF-induced MAPK activity.

The insulin-PI3K-PKB pathway is a major signalling pathway regulating multiple cellular functions and processes. It has been shown that FOXO signalling and down-regulation of this signalling by the insulin pathway has great effects on cellular behaviour. We have previously demonstrated that FOXOs can induce cell cycle arrest via multiple transcriptional mechanisms [9,10]. Furthermore, we have provided evidence that FOXO can subsequently trigger cells into quiescence, a reversible cellular state of cell cycle arrest, by increasing p130 levels [35]. In this previous study [35] we showed that the induction of a G₁ cell cycle arrest by p16^{ink4a} can increase caveolin-1 expression as well. It could be that, unexpectedly, with this result we revealed yet another way of direct control of caveolin-1 expression. p16^{ink4a} acts as an inhibitor of the cyclin D-CDK4 complex, which in turn regulates E2F transcriptional activity via phosphorylation of members of the Rb family. Thus a direct mechanism by which p16^{ink4a} may regulate caveolin-1 expression could involve E2F transcription factors. Alternatively, as we initially set out to investigate, the regulation of caveolin-1 by p16^{ink4a} is indirect and is, as such, due to the G₁ cell cycle arrest. How a G₁ arrest could result in increased caveolin-1 expression is unclear, but it is possible that changes in the composition of the lipid bilayer that are induced by G₁ cell cycle arrest [41] cause a change in caveolin-1 expression, for example as part of a compensatory response. Alternatively, this arrest may trigger some sort of stress response and it has been shown that cellular oxidative stress induces caveolin-1 expression [18]. However, in the present study we have shown that caveolin-1 levels are increased by FOXO factors through direct transcriptional control. Interestingly, we have recently obtained evidence that FOXO can be activated by cellular oxidative stress [42], suggesting that the observed stress-induction of caveolin-1 [18] may proceed via FOXO activation. The regulation of caveolin-1 expression by FOXO suggests a novel function for FOXO, namely regulation of senescence. Other groups have shown that increased caveolin-1 expression causes cell cycle arrest and senescence [17,18]. Caveolin-1 levels are high in senescent cells, but the fact that the senescent phenotype can be reverted by lowering the caveolin-1 levels suggests that the cells are not in a complete senescent state, since they have the capacity to enter the cell cycle again [20]. So we suggest that it is more likely that the FOXO-induced caveolin-1 expression is contributing to establishing quiescence rather than senescence.

It has been shown that cAMP signalling can regulate caveolin-1 levels, although it is not clear whether this involves transcriptional regulation [21]. The signalling that is induced by cAMP can also modulate cell cycle regulation. In some cell types cAMP is known to induce a cell cycle arrest, whereas in other cells cAMP can induce cell cycle progression, depending on whether cAMP inhibits or activates the ERK (extracellular-signal-regulated protein kinase) pathway respectively (reviewed in [43]). It is not known what the effect of cAMP is on cell cycle regulation in the cells used by Yamamoto and colleagues [21], but in cells that show a cAMP-induced cell cycle arrest, cAMP can inhibit phosphorylation of PKB by sequestering PDK1 (phosphoinositide-dependent kinase 1) [44]. This will lead to a subsequent activation of FOXO factors. This appears important for the cAMP-induced cell cycle arrest in cell types such as DL23 and MEF cell lines (H. B. Kuiperij, A. van der Horst, J. Raaijmakers, R. H. Medema, J. L. Bos, B. M. T. Burgering and F. J. T. Zwartkruis, unpublished work). Our findings suggest therefore a model in which the forskolin/cAMP-regulated caveolin-1 expression is through PDK1/PKB and is mediated by the activation of FOXO.

It is highly tempting to speculate on the role of FOXO-induced caveolin-1 expression with respect to tumorigenesis. However,

there is a controversy as to how caveolin-1 is related to tumorigenesis. Some studies demonstrate a correlation between increased caveolin-1 expression, tumorigenesis and metastatic capacity in various types of primary tumours such as lung adenomas and oesophageal squamous cell carcinomas [45,46]. In contrast, others have shown in several tumour cell lines and some whole tumours that caveolin-1 expression is down-regulated and that re-expression of caveolin-1 leads to inhibition of cell growth [47–49]. It is interesting to note, however, that the majority of papers that show an increase in caveolin-1 expression do so by the use of whole primary tumours, whereas the link between down-regulation of caveolin-1 and tumorigenesis is mainly shown in tumour cell lines. It could be that down-regulation of caveolin-1 in these cell lines is in fact a way to prevent replicative senescence, which is thus far only a clearly established process in cultured cells.

In conclusion, we show in the present study direct transcriptional control of caveolin-1 expression by FOXO. FOXO-mediated up-regulation of caveolin-1 expression results in attenuated EGF signalling and this may contribute to FOXO-mediated induction of cellular quiescence.

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