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The estrogen receptor α is the key regulator of the bifunctional role of FoxO3a transcription factor in breast cancer motility and invasiveness

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Abstract

The role of the Forkhead box class O (FoxO)3a transcription factor in breast cancer migration and invasion is controversial. Here we show that FoxO3a overexpression decreases motility, invasiveness, and anchorage-independent growth in estrogen receptor α-positive (ERα+) cancer cells while eliciting opposite effects in ERα-silenced cells and in ERα-negative (ERα−) cell lines, demonstrating that the nuclear receptor represents a crucial switch in FoxO3a control of breast cancer cell aggressiveness. In ERα+ cells, FoxO3a-mediated events were paralleled by a significant induction of Caveolin-1 (Cav1), an essential constituent of caveolae negatively associated to tumor invasion and metastasis. Cav1 induction occurs at the transcriptional level through FoxO3a binding to a Forkhead responsive core sequence located at position −305/−299 of the Cav1 promoter. 17β-estradiol (E2) strongly emphasized FoxO3a effects on cell migration and invasion, while ERα and Cav1 silencing were able to reverse them, demonstrating that both proteins are pivotal mediators of these FoxO3a controlled processes. In vivo, an immunohistochemical analysis on tissue sections from patients with ERα+ or ERα− invasive breast cancers or in situ ductal carcinoma showed that nuclear FoxO3a inversely (ERα+) or directly (ERα−) correlated with the invasive phenotype of breast tumors. In conclusion, FoxO3a role in breast cancer motility and invasion depends on ERα status, disclosing a novel aspect of the well-established FoxO3a/ERα interplay.

Therefore FoxO3a might become a pursuable target to be suitably exploited in combination therapies either in ERα+ or ERα− breast tumors.

Introduction

Breast cancer is one of the most common forms of cancer observed in women. Endogenous estrogens are thought to play a major role in its development and estrogen receptor blockers are important drugs in its treatment *(Pike MC et al., 1983; Mc Guire WL, 1978; Fisher B et al.,2001).* It has been shown that longer exposures to estrogens result in an increased risk for breast cancer *(Henderson IC, 1993).* Estrogens have effects on many organ systems, beyond the reproductive system, in both females and males. Breast tissue is particularly dependent on circulating estrogens since there is no breast development in aromatase-deficient women and estrogen therapy of these patients leads to normal pre- and post-pubertal breast development. Estrogen effects are exerted through two types of specific receptor: estrogen receptor alpha (ERα) and beta (ERβ) *(Korach KS, 1994; Gustafsson JA, 1999; Dupont S et al, 2000)*. These nuclear receptors are ligand-dependent transcription factors that mediate the biological effects of estrogens and anti-estrogens. The human estrogen receptors ERα and ERβ contain five functional domains (A–E) as other members of the nuclear receptor superfamily and an additional domain F in their C terminal part *(Kumar V et al, 1987; McKenna NJ et al,1999; Shiau AK et al,1998; Brzozowski AM et al,1997; Moras D et al, 1998; Katzenellenbogen BS et al, 2002; McDonnell DP et al, 2002; MacGregor JI et al; Kato S, 1995).* The binding of estrogen in the hormone-binding domain (HBD) induces a trans-conformational change of the whole molecule allowing unmasking of the activating function 1 (AF1) in domain A/B by removal of chaperone (HSP90), dimerization, activation of activating function 2 (AF2) in the C-terminal part of the E domain and binding to estrogen-responsive element (ERE) on DNA via domain C. Estrogen receptors act mainly by regulating the expression of target genes whose promoters contain specific sequences called estrogen-responsive element (ERE). After ERE-binding of ligand-bound ER dimers, modulation of transcription occurs via interaction with coactivators or corepressors. All

together, these complexes play an important role in the recruitment of transcriptional machinery, the modulation of chromatine structure, and then in the regulation of ER target-gene expression *(McKenna NJ et al, 1999)*. The ER conformation differs with the type of ligand, and there is a marked difference in the topology of the ER surface between agonist and antagonist-bound receptors *(Shiau AK et al, 1998; Brzozowski AM et al, 1997; Moras D et al, 1998)*. Moreover, studies conducted with synthetic anti-estrogens, such as tamoxifen, have shown that the agonist/antagonist profile of a ligand varies with the tissue and the target gene considered. This led to the term of selective estrogen receptor modulators (SERMs) to define this class of drug *(Katzenellenbogen BS et al, 2002; McDonnell DP et al, 2002; MacGregor JI et al,1998)*. ER activity can also be modulated through indirect activation of the ER by growth factors or cytokines independently of the binding of natural or synthetic hormones *(Kato S et al, 1995; Bunone G et al, 1996)*. Estrogen and progesterone receptors (ERα and PR) have now been studied in clinical breast cancer for more than 20 years. ERα was found in 50–80% of breast tumors and ERα status is essential in making decisions about endocrine therapy *(Mc Guire WL, 1978; Osborne CK,1998)*. Positive receptor status correlates with favorable prognostic features, including a lower rate of cell proliferation and histologic evidence of tumor differentiation. During the first several years after diagnosis, patients with ER-positive tumors tend to have a lower recurrence rate; however, this is balanced by a higher recurrence rate in subsequent years so that the overall prognostic significance of receptor status is modest. ERα and PR have their greatest utility in predicting response to hormonal therapy, both in the adjuvant setting and for advanced disease. Tumors that express both ERα and PR have the greatest benefit from hormonal therapy, but those containing only ERα or PR still have significant responses *(Bardou VJ et al, 2003)*. Does the ER-negative tumor derive from ER-positive tumor or is it a totally different disease *(Zhu K et al,1997)*? This question remains open since differences in ERα expression

appear in tumors as early as carcinoma in situ *(Roger P et al, 2000)* and the gene expression is substantially different in the two types of invasive carcinoma *(Sheikh MS et al, 1994; Thompson EW et al, 1992)*. Moreover, ERα re-expression in an ERα-negative cancer cell is not sufficient to restore the ERα-positive phenotype, particularly in terms of mitogenic response and the pattern of gene expression *(Garcia M et al, 1992; Jiang SY et al,1992)*. The great interest on ERα is also due to its functional cross-talk with other factors such the forkhead box class protein members.

Forkhead proteins are not among the largest transcription factor families, but display a remarkable functional diversity and are involved in a wide variety of biological processes. The name is derived from the two spiked-head structures in the embryos of the *Drosophila fork head* mutant, which are defective in anterior and posterior gut formation *(Weigel D et al,1989)*. With the 1990 discovery of a 110-amino-acid DNA binding domain that was almost perfectly conserved between FORK HEAD and the mammalian HNF-3 transcription factors, it became clear that this motif defined a novel transcription factor family *(Weigel D et al,1990)*. Among the organisms for which the genome sequences are completed, or nearly so, there is indeed a correlation between anatomical complexity and forkhead gene number: 4 in *Saccharomyces* and *Schizosaccharomyces*, 15 in *Caenorhabditis*, 20 in *Drosophila,* and 39 in *Homo*. In 2000, the nomenclature of chordate forkhead transcription factors was revised *(Kaestner KH,2000; Kaestner KH et al,2000)*. The new nomenclature, which uses Fox (for "Forkhead box") as the root symbol, ensures that the same name is used for orthologous genes in different species and reflects phylogenetic relationships by including a letter that indicates subfamily. Within a subfamily, each gene is identified by a number (e.g., *FoxO2*), the typography follows the conventions used in each species (*FOXO3a* in *Homo, Foxo3a* in *Mus*, and *FoxO3a* in all others), and proteins are distinguished from genes by the use of roman type (e.g., FoxO3a). Forkhead proteins, the transcription factors of

wingedhelix domain, are characterized by a conserved DNA-binding domain—the forkhead box among invertebrate and mammalian cells *(Weigel D et al,1989; Weigel D,1990; . Kaufmann E et al,1996)*.

Based on the forkhead box domain, the forkhead genes are grouped into 19 subclasses of Fox genes *(Kaestner KH,2000; Kaestner KH et al,2000)*. FoxO transcription factors, one of largest subgroups of forkhead family members, are characterized by a conserved DNA-binding domain-the forkhead box among invertebrate and mammalian cells *(Arden KC,2006; Greer EL et al,2005)*. The FoxO subfamily contains four members (FoxO1, FoxO3, FoxO4, and FoxO6), which activate or repress multiple genes such as Bim and FasL involved in apoptosis *(Finnberg N et al,2004; Tran Het al,2003)*, p27kip *(Dijkers PF et al,2000)* and cyclin D *(Schmidt M et al,2002)* in cell cycle regulation, GADD45a in DNA damage repair *(Greer EL et al,2005; Finnberg N et al,2004; Tran Het al,2003; Yang JY et al,2006)*, manganese superoxide dismutase (MnSOD) in stress response *(Kops GJ et al,2002)*, and glycogenolytic gene glucose-6-phosphatase (G6pc) in metabolism *(Onuma H et al,2006)*. Recent studies also reveal the importance of FoxOs in preserving the self renewal capacity of hematopoietic stem cells *(Miyamoto K et al,2007; Tothova Z et al,2007)*, however, the detailed mechanisms are currently a work in progress.

The major consequence of the phosphorylation of FoxO transcription factors by Akt and SGK is a change in the subcellular localization of these transcription factors *(Biggs et al., 1999; Brunet et al., 1999; Takaishi et al., 1999)*. In the absence of growth factors, when Akt and SGK are inactive, FoxO factors are localized within the nucleus. When cells are exposed to growth factors, the PI3K–Akt/SGK cascade is activated and triggers the export of FoxO factors to the cytoplasm. Mutation analyses have revealed that one or two leucine-rich domains in the conserved C-terminal region of FoxO proteins function as a nuclear export sequence (NES) *(Biggs et al., 1999; Brunet et al., 2002)*. In addition, phosphorylated FoxO factors have been shown to specifically interact

with 14-3-3 proteins, which serve as chaperone molecules to escort FoxO proteins out of the nucleus *(Brunet et al., 1999, 2002)*. Several mechanisms have been proposed to explain how 14-3-3 binding to FoxO factors promotes the relocalization of FoxO factors from the nucleus to the cytoplasm. While 14-3-3 proteins are mostly present in the cytoplasm at equilibrium, these chaperone molecules have been found to bind to their substrates in the nucleus *(Brunet et al., 2002)*. Consistently, 14-3-3 binds to FoxO3 in the nucleus *(Brunet et al., 2002)*. 14-3-3 binding may decrease the ability of FoxO factors to bind DNA, releasing FoxO proteins from a nuclear DNA anchor *(Cahill et al., 2000)*. 14-3-3 binding to FoxO factors may actively promote the nuclear export of FoxO factors, perhaps by inducing a conformational change in FoxO molecules that would expose the NES and allow interaction with Exportin/Crm1 *(Brunet et al., 2002)*. 14-3-3 binding to FoxO factors may also prevent the nuclear reimport of these transcriptional regulators by masking FoxO nuclear localization signal (NLS) *(Brownawell et al., 2001; Rena et al., 2001)*. Finally, the phosphorylation of FoxO factors at Ser322 and Ser325 appears to accelerate FOXO relocalization to the cytoplasm in response to growth factors by increasing the interaction between FoxO and the export machinery (Ran and Exportin/Crm1) *(Rena et al., 2002)*. These various mechanisms for regulating the translocation of FoxO transcription factors from the nucleus to the cytoplasm may serve as a fail-safe mechanism to ensure a complete sequestration of FoxO factors away from their target genes. Mutational analysis of the three regulatory Akt/SGK sites have revealed that the phosphorylation of each site contributes to the nuclear exclusion of FoxO factors *(Brunet et al., 2001)*. One attractive possibility is that each site participates in different aspects of the mechanisms that ensure the relocalization of FoxO proteins into the cytoplasm. Thus, phosphorylation of FoxO factors may represent a way of modulating the extent of the relocalization of these transcription factors to the cytoplasm in different cell types or in response to different combinations of signals. The most recently

identified FoxO member, FoxO6, only contains two of the three Akt/SGK regulatory sites (Thr26 and Ser184 in mouse FOXO6) *(Jacobs et al., 2003)*. Unlike the other FoxO isoforms, FoxO6 is mostly nuclear. However, FoxO6 phosphorylation at Thr26 and Ser184 appears to decrease the transcriptional activity of this FoxO isoform *(van der Heide et al., 2005)*. These findings suggest that the regulations and functions of FoxO6 may differ from those of FoxO1, FoxO3, and FoxO4.

The protein phosphatases that dephosphorylate FoxO transcription factors at the sites that are targeted by Akt and SGK remain elusive. These phosphatases would have the capacity to counteract Akt/SGK actions and to rapidly activate FoxO proteins, by allowing these transcription factors to translocate to the nucleus.

As FoxO factors appear to play an important role in cell cycle arrest, identifying ways to activate FoxO factors may be critical to counteract tumor formation.

While FoxO transcription factors are mainly regulated via reversible changes in subcellular localization, the degradation of FoxO protein represents an additional and irreversible level of regulation of this family of transcription factors. FoxO protein degradation often accompanies cell transformation *(Hu et al., 2004; Huang et al., 2005)*, suggesting that this mechanism of regulation may be a critical initiation step towards tumorigenesis.

The degradation of FoxO transcription factors is mediated by the ubiquitin–proteasome pathway *(Matsuzaki et al., 2003; Plasand Thompson, 2003; Aoki et al., 2004; Hu et al., 2004; Huang et al., 2005)*.

Akt activity is necessary for ubiquitin-mediated degradation of FoxO3 and FoxO1 (*Plasand Thompson, 2003; Huang et al., 2005)*. In addition, I kappaB kinase β (IKKβ) also causes the proteasome-dependent degradation of FoxO factors *(Hu et al., 2004)*. IKKβ is known to activate the transcription factor NF-kB through the phosphorylation and subsequent degradation of IkB, which normally serves as a negative regulator of NF-kB *(Karin et al., 2002)*. IKKβ

induces the phosphorylation of FoxO3 at Ser644, in the extreme C-terminal portion of the molecule. This phosphorylation results in the ubiquitination and subsequent degradation of FoxO3 *(Hu et al., 2004)*. Since IKKβ-induced tumorigenesis can be suppressed by overexpression of FoxO3 *(Hu et al., 2004)*, the regulation of FoxO protein degradation by IKKβ may play an important role in tumorigenesis. However, Ser644 is not conserved in other FoxO isoforms and is not present in worms and flies. Thus, whether IKKβ phosphorylates and controls the other FoxO isoforms remains to be determined. It is possible that the degradation of FoxO isoforms is regulated by different protein kinases via independent mechanisms. FoxO1 and FoxO3 protein degradation is regulated by Akt and, at least for FoxO3, by IKKβ. However, whether FoxO4 and FoxO6 protein degradation is also actively regulated, and if so, whether the mechanisms of regulation are similar, still remains to be established. One major difference between the FoxO family members is that they display overlapping but different patterns of expression. While these differences may be partly due to mRNA expression, it is possible that protein degradation also plays an important role in the distinction between FoxO isoforms in vivo. As tumorigenesis appears to be associated with a loss in FoxO proteins, understanding the regulation of FoxO expression will likely give important insight into mechanisms that govern tumor suppression.

An increasing interest in FoxOs factors has been lately observed in the oncologic research field. In particular, in breast cancer, its role is still controversial, in fact, FoxO3a overexpression has been shown to inhibit tumor growth in vitro and to reduce tumor size in vivo, *(Hu MC et al, 2004; Yang JY et al, 2008; Zou Y et al, 2008)* and cytoplasmic location of FoxO3a seems to correlate with patients poor survival *(Hu MC et al, 2004)*. Moreover, genetic deletion of the FoxOs alleles (FoxO1a, FoxO3a, and FoxO4) generates progressive cancerous phenotypes, such as thymic lymphomas and hemangiomas. These data elucidate FoxOs as bona fide tumor suppressor genes

(Paik JH, 2007). Additionally, FoxO members seem to be important mediators of the well-established functional cross-talk between estrogens and growth factors, which play a pivotal role in breast cancer development and progression *(Sisci D et al,2007)*.

In fact, growth factors are known to influence the expression and activity of estrogen receptor α (ERα) and its transcriptional cofactors; conversely, ERα regulates the expression of growth factor receptors and their ligands and signaling intermediates *(Lanzino M et al, 2008)*. In this context, several reports have recently suggested a functional interaction between ERα and FoxO members.

17β-estradiol (E2) has been noted to determine ERα binding to FoxO1a, FoxO3a, and FoxO4, which, in turn, showed either coactivator or corepressor functions on estrogen-responsive element (ERE) sites, depending on the cellular model *(Zou Y et al, 2008; Schuur ER et al, 2001; Zhao HH, 2001)*. Moreover, we introduced the importance of Akt2/FoxO3a axis in the control of ERα-mediated transcription in ERα-positive (ERα+) breast cancer cells. Our results indicate that Akt2 inhibition reduces ERα transcriptional activity through FoxO3a activation, suggesting that FoxO3a, acting as a co-repressor for ERα, could exert a protective role in ERα+ breast tumors *(Morelli C, 2010)*.

In line with this assumption, Belguise et al. showed that ectopic expression of a constitutively active FoxO3a overrode transforming growth factor-B1-mediated invasive phenotype and induced a more epithelial phenotype in ERα+ mouse mammary tumors *(Belguise K et al, 2007)*. However, more recently, FoxO3a has been described to behave in an opposite fashion in several other cancer cell lines, which, interestingly, were all ERα-negative (ERα-); in fact, Storz et al. reported that, in tested cells, nuclear retention of FoxO3a resulted in greatly increased invasion, through the induction of matrix metalloproteinase 9 (MMP-9) and MMP-13*(Storz P et al, 2009)*. Due to the inconsistency of the data available from ERα+ and ERα− breast cancer cells, the interplay between ERα

and FoxO3a in tumor metastasis needs further investigations and is the goal of the present study. Since it is well documented that, in breast cancer, ERα signaling strongly correlates with a lower invasiveness and reduced metastatic potential, *(Rochefort H et al, 1998)* we assume that FoxO3a/ERα interplay could be responsible for the reduction of the migrating and invasive phenotype only in ERα+ cells, while, in ERα− cells, the lack of the α isoform of the receptor might enable FoxO3a to act in an opposite fashion. In ERα+ cells, FoxO3a-mediated events were paralleled by a significant induction of Caveolin-1 (Cav1), an essential constituent of caveolae negatively associated to tumor invasion and metastasis. Caveolae demarcate discrete, highly ordered microdomains of the plasma membrane that serve as dynamic trafficking and signal transduction sub-compartments. Caveolae were originally described morphologically in 1953-1955 as flask-shaped invaginations of the plasma membrane with a diameter of 50-100 nm in endothelial and epithelial cells *(Bruns RR et al, 1968)*. Caveolae occur as both invaginations of the plasma membrane proper and as detached vesicles residing close to the membrane. Caveolae exist in numerous tissues and cell types with varying abundance, the highest levels occurring in fibroblast, adipocytes, endothelial cells, type 1 pneumocytes, epithelial cells and smooth and striated muscle cells *(Engelman JA et al,1998; Scherer PE et al,1995; Scherer PE et al, 1997)*. By electron microscopy caveolae charatteristically appear smooth with a distinctive coat appearing as bipolar-oriented, thin striations surrounding the bulb of the caveolae *(Rothberg KG et al, 1992; Peters KR, 1985)*. The striated coat associated with the cytoplasmatic face of caveolae is principally formed by homo- and hetero-oligomers of the structural coat proteins the caveolins (caveolin1,2,3) *(Scherer PE et al, 1997; Monier S et al, 1995; Sargiacomo M et al, 1995; Tang Z et al, 1996)*. As a protein family, caveolins can be defined as cytoplasmatic membrane proper and participate in the sequestration of inactive signaling molecules. As caveolin-1 was the first caveolin member to be identified it has served as the prototype for the study of

caveolins. Based on primary sequence composition and mutation analysis caveolin-1 is predicted to have a central 33 amino acid hydrophobic domain (residues 102-134), thought to form a hairpin structure spanning the membrane, with the hydrophilic amino (1-101) and carboxyl termini (135-178) remaining cytoplasmatic *(Monier S et al, 1995; Sargiacomo M et al, 1995; Dupree P et al, 1993).*

Thus, the present work was aimed to undertake an accurate study on the molecular mechanisms through which FoxO3a regulates migration and invasion in ERα+ breast cancer cells. Our results offer new interesting insights on FoxO3a activity, elucidating additional mechanisms that could represent novel targets in breast cancer therapy.

Results

Cell motility, invasion, and anchorage-independent growth are inhibited in ERα+ breast cancer cells overexpressing FoxO3a

To assess the role of FoxO3a in the metastatic and invading potential of breast cancer cells, wild-type FoxO3a (F3a) was overexpressed in ERα+ MCF-7. Our results show a significant reduction of migrating and invading MCF-7/F3a cells (***Fig. 1A and B***), compared with control samples. Ectopic expression of the constitutively active triple mutant of FoxO3a (F3aAAA), where the 3 known PKB phosphorylation sites have been mutated to alanine, so that FoxO3a can no longer be inhibited by PKB-mediated phosphorylation, emphasized the phenomenon (***Fig. 1A and B***), suggesting that FoxO3a modulation of the migrating and the invading potential could involve the transcriptional induction of Forkhead responsive genes. FoxO3a silencing (siF3a) confirmed these data, since it led to a substantial increase in cell migration and invasion (***Fig. 1A and B***). Moreover, in agreement with our previous observations, *(Sisci D et al, 2010)* E2 treatment strongly reduced motility and invasion, and the effect was additive in F3a- and F3aAAAoverexpressing samples, while siF3a only in part was able to counteract E2-mediated effects (***Fig. 1A and B***).

In addition, anchorage independence, a characteristic of malignancy and tumor progression , was also investigated in F3aoverexpressing and silenced MCF-7 cells through soft agar colony formation assay. We observed a dramatic decrease of the number as well as of the dimensions of the colonies in MCF-7/F3a samples, reaching almost completely the condition of single cells in F3aAAA-expressing cells (***Fig. 1 C_1 and C_2***). The same trend was evidenced in E2-treated samples, showing how FoxO3a, especially in its active form, is able to counteract the well-known positive effect of the nuclear hormone on the colony formation of MCF-7 cells *(Manni A et al, 1991).* As expected, an increase in the number of colonies was observed following siF3a, and such increase became

more evident in presence of E2 (***Fig.1 C_1 and C_2***). Transfections and silencing efficiency were assessed on total protein lysates (***Fig. 1D***).

Interestingly, F3a and F3aAAA overexpression in other ERα- positive cell lines, ZR-75 (breast cancer) and Ishikawa (endometrial cancer), led to results that were comparable to those obtained from MCF-7, both in presence or absence of E2 (***Fig.2, upper panels***)

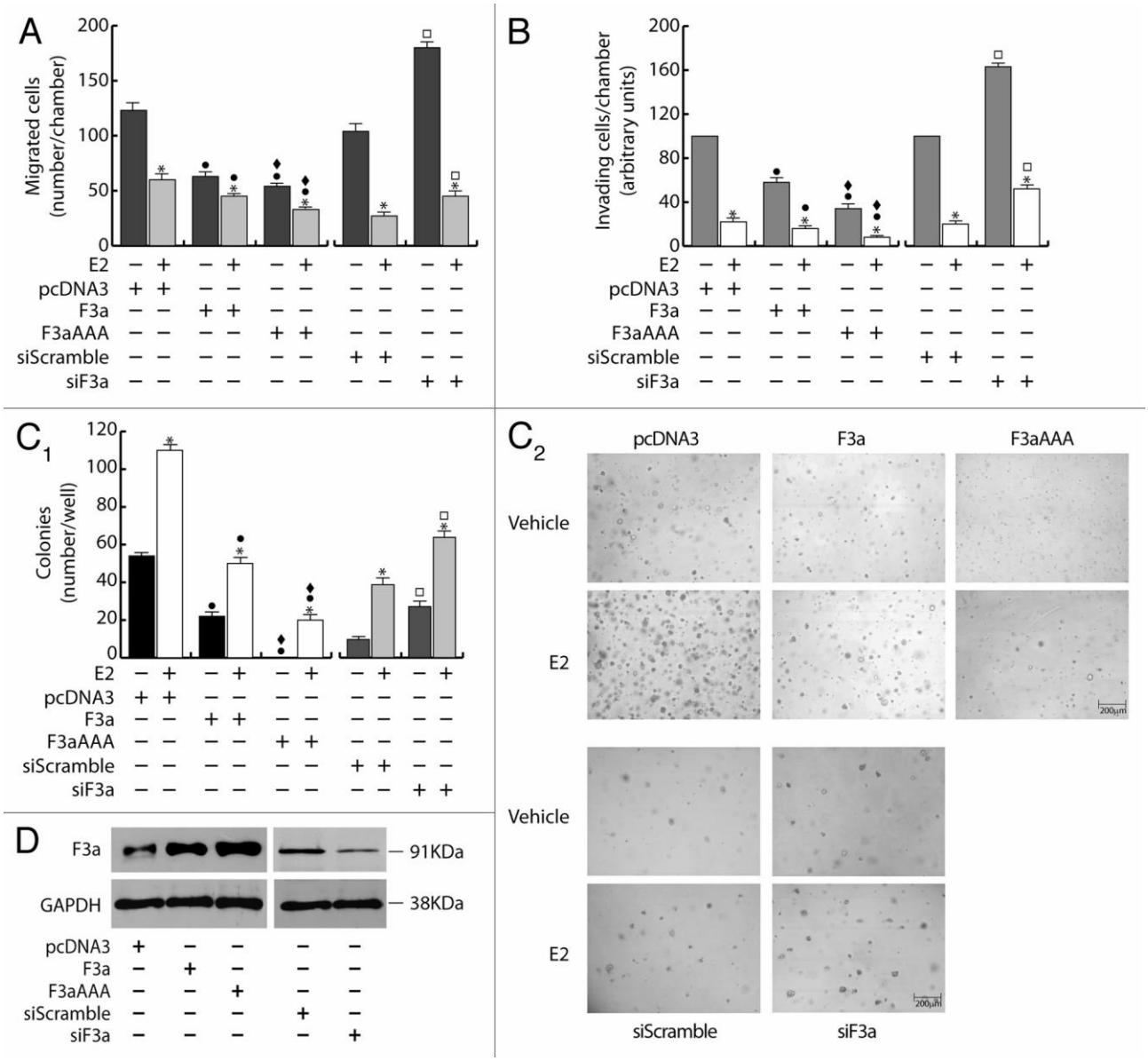


Figure 1. FoxO3a inhibits migration, invasion and anchorage independent growth in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells was transiently transfected with 1 μg/35 mm dish of F3a, F3aAAA, or pcDNA3 as control. Another double set was silenced for FoxO3a expression (siF3a),using a siScramble as control (60 pmol siRNAs/35 mm dish). After 5 h cells were switched to PRF-SFM, and the next day one of each set of cells was harvested and subjected to migration (**A**), invasion (**B**), and soft agar assay (**C1 and C2**). Migration and invasion assays were conducted as described in "Materials and Methods", adding 100 nM E2 in the bottom of the wells where indicated. Migrated and invading cells were evaluated after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies >50 μm diameter formed after 14 d from plating were

photographed at 4× magnification (**C2**) and counted under the microscope (**C1**). The second set of either transfected or silenced MCF-7 cells was used for total protein extractions and WB analysis to assess transfections efficiency; GAPDH was evaluated as a loading control (**D**). Results are reported as the mean ± s.d. of at least 3 independent experiments. In all experiments, significance values were as follows: *, $P < 0.01$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦,$P < 0.05$ vs. corresponding F3a; □,$P < 0.01$ vs. corresponding siScramble.

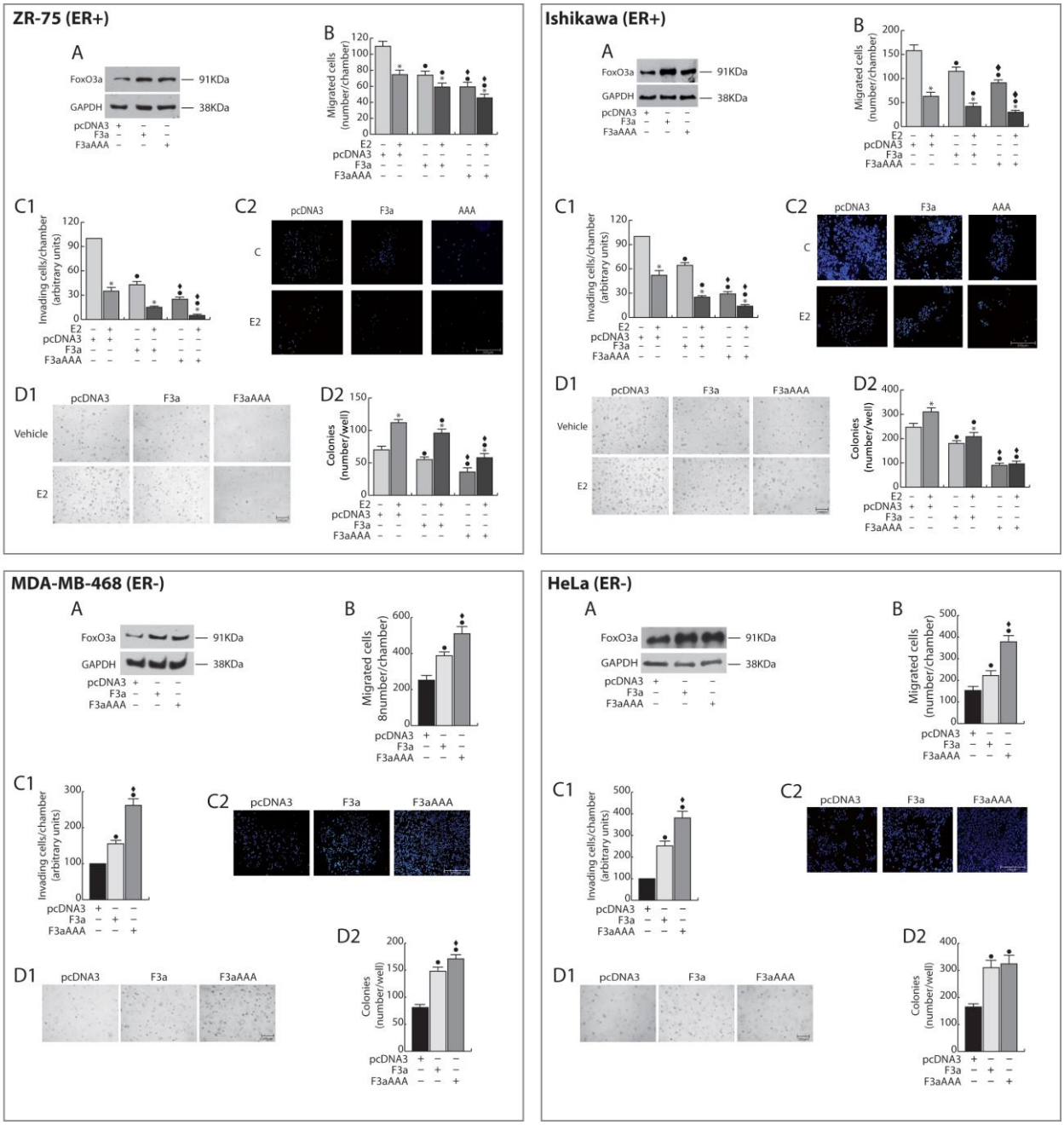


Figure 2 The opposite effects exerted by FoxO3a on migration, invasion and colony formation of ERα+ and ERα cancer cells is not tissue specific.

Double sets of ERα+ (ER+) breast cancer cells (ZR75) and endometrial cancer cells (Ishikawa), and of ERα (ER)breast cancer cells (MDAMB468) and cervical cancer cells (HeLa) were transiently transfected with F3a, F3aAAA or pcDNA3 as described in Materials and Methods. Following 24h of starvation, one of each double set of cells was harvested and subjected to Migration (**B**), invasion (**C1 and C2**) or soft agar assays (**D1 and D2**), adding 100nM E2 where indicated. Migrated cells were counted after 24h (ZR72 and Ishikawa) or 16h (MDAMB468 and HeLa) of incubation. Invading cells were counterstained with DAPI after 48h (MDAMB468 and HeLa) or 72h (ZR75, Ishikawa) of incubation (**C2**), and evaluated by ImageJ software (**C1**). In soft agar assays, colonies formed after 14 days from plating were photographed at 4x magnification (**D1**), exposed to

MTT and counted under the microscope (**D2**). The second of each set of cells was used to evaluate transfections efficiency by WB analysis on total protein extract; GAPDH was used as a loading control (**A**). Results are the mean ±s.d. of at least three independent experiments. *, P < 0.01 vs untreated; ●,P < 0.01 vs pcDNA3;♦,P < 0.01 vs F3a.

The lack of ERα reverses FoxO3a-mediated inhibition of migration, invasion, and colonies formation

To assess if the effects of FoxO3a on motility, invasiveness, and colony formation could depend on ERα, silencing experiments were conducted in MCF-7, using specific siRNAs against ERα (siER) (***Fig. 3***). Interestingly, ERα silencing was able to counteract FoxO3a-mediated inhibition of the above-mentioned pathological features.

In particular, compared with control (siScramble), siER led to an increase in cell migration and invasion, which became even more evident in F3a and, especially, in F3aAAA-expressing cells (***Fig. 3A and B***), confirming that ERα is a hallmark of a less motile and invading phenotype, *(Sisci D et al, 2010; . Platet N et al, 2004)* and that FoxO3a's effect on cell motility and invasiveness can switch from inhibitory to stimulatory, depending on the presence or absence of ERα, respectively. Moreover, in siER samples, reasonably due to the lack of the receptor, E2 treatment no longer caused the reduction of the invading potential of MCF-7 (***Fig. 3B***) and even showed the opposite effect on cell motility, which rather increased over the respective controls (***Fig. 3A***). These evidences suggest that, in absence of a functional ERα, E2 could trigger some other pathway that stimulates cell migration (although not invasion), and that FoxO3a can somehow cooperate with the hormone in this process.

As expected, ERα silencing was able to inhibit both basal and E2 induced MCF-7 growth in soft agar by strongly reducing the number and the dimensions of colonies compared with non-treated and E2-treated siScramble samples, respectively (***Fig. 3C***). However, as in migration and invasion experiments, the inactivation of the nuclear receptor reversed the effect of ectopic F3a and F3aAAA, which, either in absence or presence of E2 treatment, induced an

increase in the number of colonies, instead of the decrease observed in siScramble samples (***Fig. 4C***).

The fact that ERα exerts a pivotal role in determining FoxO3a behavior was confirmed by the results obtained in ERα− cells. Indeed, overexpression of FoxO3a in ERα− breast cancer MDA-MB-231 cells was able to induce an evident increase (rather than a decrease, as in ERα+ cells) of the migrating and invading potential (***Fig. 4A and B***), as well as, when grown in soft agar, F3a-overexpressing cells formed many more and larger colonies compared with control vector (***Fig. 4C$_1$ and C$_2$***). Once again, in all experiments, F3aAAA was more effective than F3a, while an evident reduction of migration, invasion and number and dimensions of colonies was observed in F3a silenced samples (***Fig. 4A–C$_2$***). Transfections and silencing efficiency were determined concomitantly (***Fig. 4D***).

Noteworthy, as in MDA-MB-231, F3a and F3aAAA overexpression led to comparable results in other ERα− breast cancer cell lines (MDA-MB-468 and MDA-MB-435) as well as in ERα− cervical cancer HeLa cells, indicating that FoxO3a functions trough mechanisms that are not tissue-specific (***Fig.2, lower panels and data not shown***).

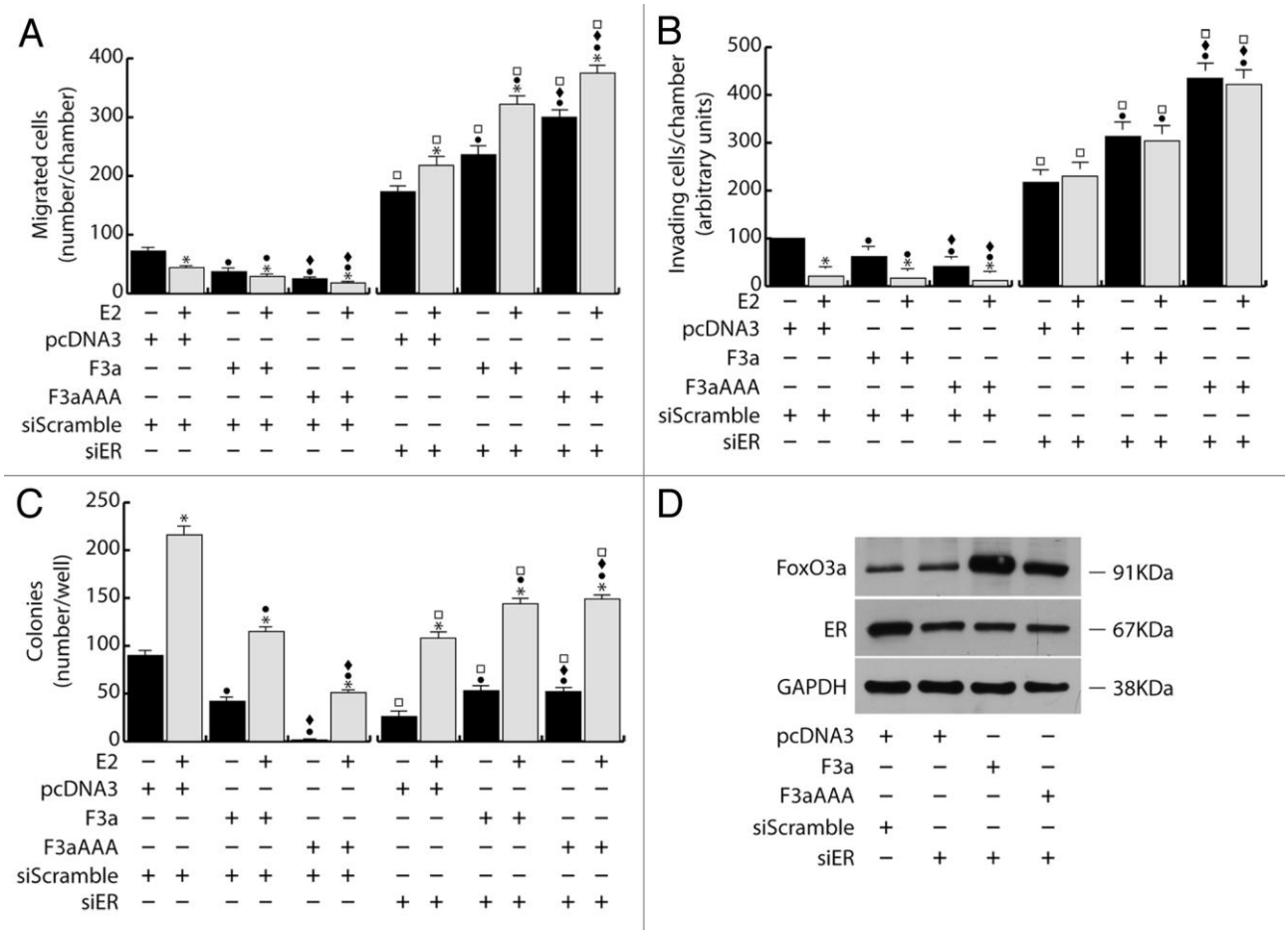


Figure 3. FoxO3a mediated inhibition of breast cancer cell migration, invasion and growth in suspension depends on ERα. Two double sets of MCF-7 cells were silenced either for ERα (siER), using siScramble as control. After 5 h cells were switched to PRF-SFM and transiently transfected with F3a, F3aAAA, or pcDNA3. Next day cells were harvested and one set of each experiment was subjected to migration, invasion, and soft agar assay in the presence or in the absence of E2. Migrated (**A**) and invading (**B**) cells were evaluated after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies ≥50 μm diameter formed after 14 d from plating were counted under the microscope (**C**). The second set of each experiment was used for total protein extraction to evaluate transfections efficiency by WB analysis; GAPDH was used as loading control (**D**). Results are the mean ±s.d. of at least three independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦, $P < 0.01$ vs. corresponding F3a; □ , $P < 0.01$ vs. corresponding siScramble.

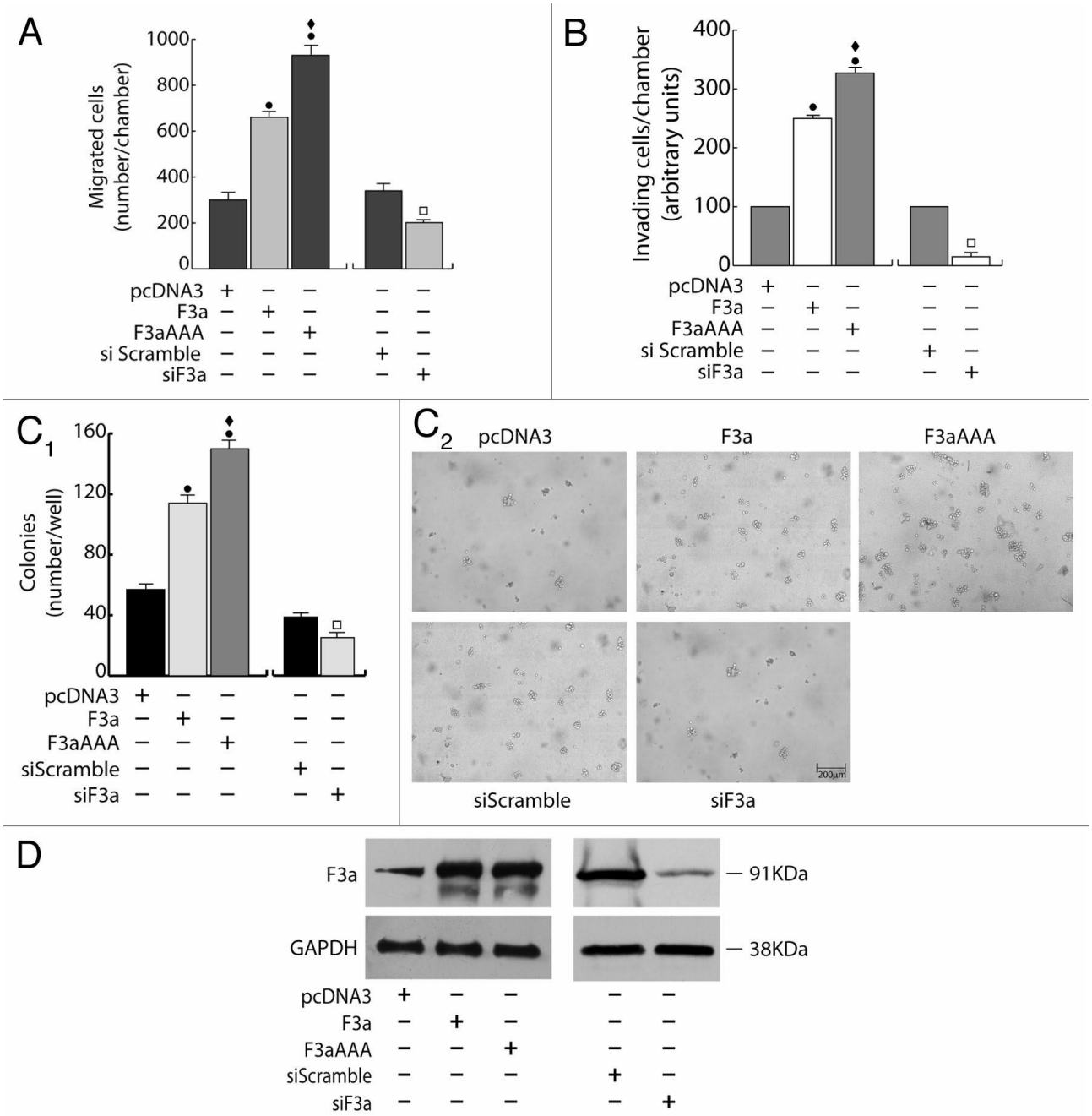


Figure 4. FoxO3a promotes migration, invasion, and anchorage-independent growth in ERα− MDA-MB-231 breast cancer cells. A double set of MDA-MB-231 cells were transiently transfected with 1 µg/35 mm dish of F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a expression (siF3a) using a siScramble as control (60 pmol siRNAs/35 mm dish). Both transfection and silencing were made on cells in suspended PRF-GM. After 5 h cells were serum starved and, 24 h later, harvested. One set was subjected to migration (**A**), invasion (**B**), or soft agar assay (**C1 and C2**). Migrated and invading cells were evaluated after 16 h and 48 h of incubation, respectively. In soft agar assay, colonies > 50 µm diameter formed after 14 d from plating were photographed at 4× magnification (**C2**) and counted under the microscope (**C1**). The second set of either transfected or silenced MCF-7 cells was used to assess transfections efficiency by WB analysis on total protein extracts; GAPDH was evaluated as a loading control (**D**). Results are reported as the mean ± s.d. of at least 3 independent experiments. ●, $P < 0.01$ vs. pcDNA3; ♦, $P < 0.01$ vs. F3a; □ , $P < 0.05$ vs. siScramble.

FoxO3a and E2 synergistically induce caveolin-1 expression in ERα+ cancer cells

To the aim of identifying the mechanism through which FoxO3a modulates cell motility and invasiveness, we focused our attention on caveolin-1 (Cav1), a protein that has been reported to be induced by both Forkhead transcription factors *(van den Heuvel AP et al, 2005)* and E2 *(Charpentier AH et al, 2000; Razandi M, 2002)*.

Since, in breast cancer, Cav1 has been negatively *(Sloan EK et al, 2004)* and positively *(Joshi B et al, 2008)* linked to tumor progression, motility, and invasiveness, we questioned if FoxO3a could control migration and invasion of breast cancer cells through the modulation of Cav1 expression.

In ERα+ MCF-7 cells, the ectopic expression of FoxO3a caused a strong upregulation of Cav1 protein and mRNA, which was even more evident in F3aAAA transfectants, suggesting that FoxO3a induction of Cav1 expression could occur at the transcriptional level. As expected, E2 treatment increased Cav1 levels, and the effect was additive to that exerted by F3a or F3aAAA (***Fig.5A and B***). Silencing experiments confirmed FoxO3a involvement in Cav1 transcription, leading to a decrease in Cav1 content and attenuating the E2-dependent Cav1 induction (***Fig. 5C and D***). Notably, Cav1 undergoes similar regulation by E2 and FoxO3a in the other 2 tested ERα+ cell lines, ZR-75 and Ishikawa (***Fig. 6***). In particular, the induction of Cav1 by E2 is ERα- dependent, since (1) the pure antiestrogen ICI 172.780 was able to abrogate the effect of E2 on Cav1 expression in ERα+ MCF-7 cells (***Fig. 5E***); and (2) the hormone did not increase Cav1 expression in ERα−, although ERβ+, MDA-MB-231 cells (***Fig. 5F***).

In light of these evidences we could hypothesize that, in ERα+ cells, FoxO3a might promote a less aggressive phenotype by cooperating with the hormone receptor in *CAV1* gene induction.

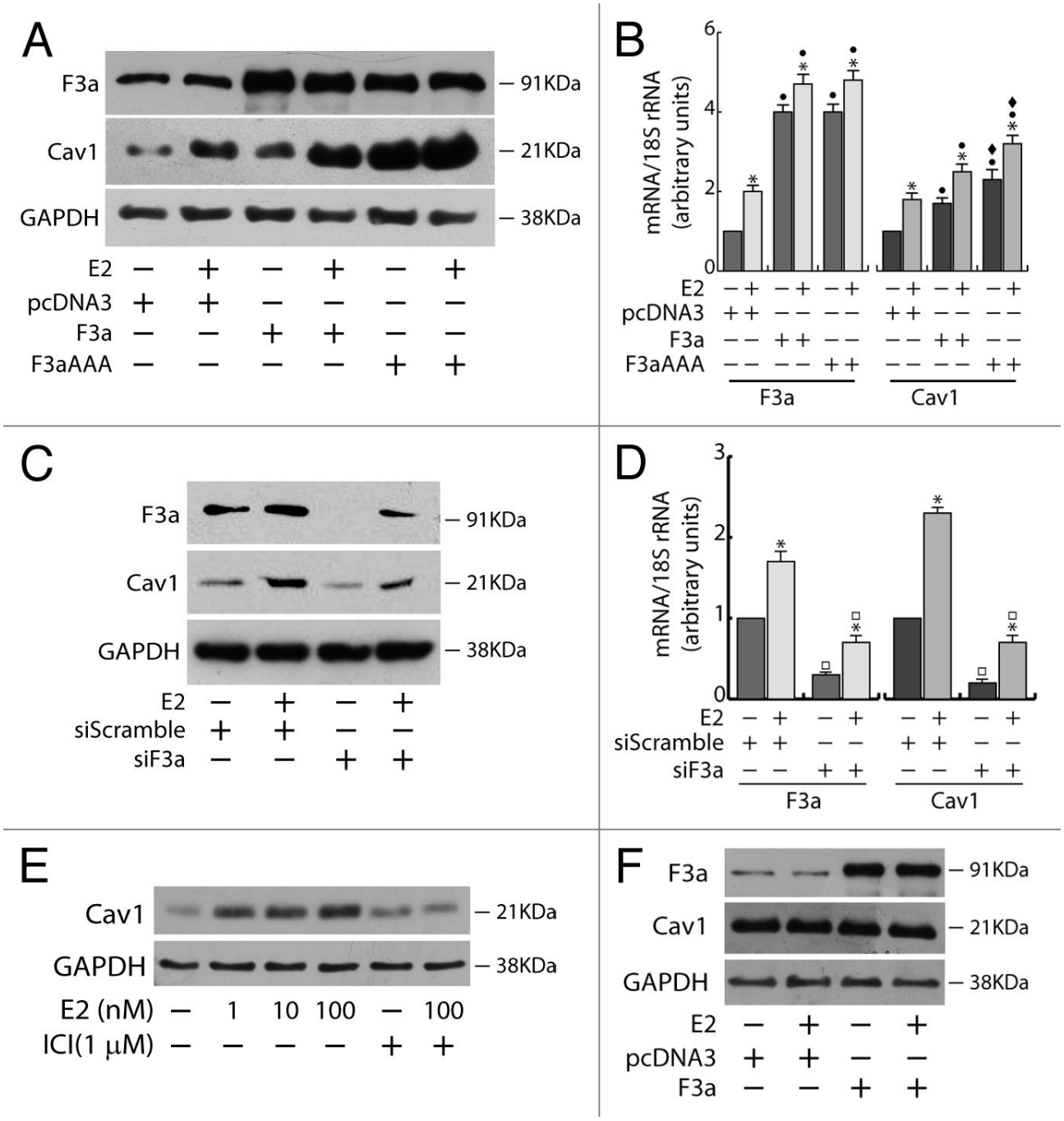
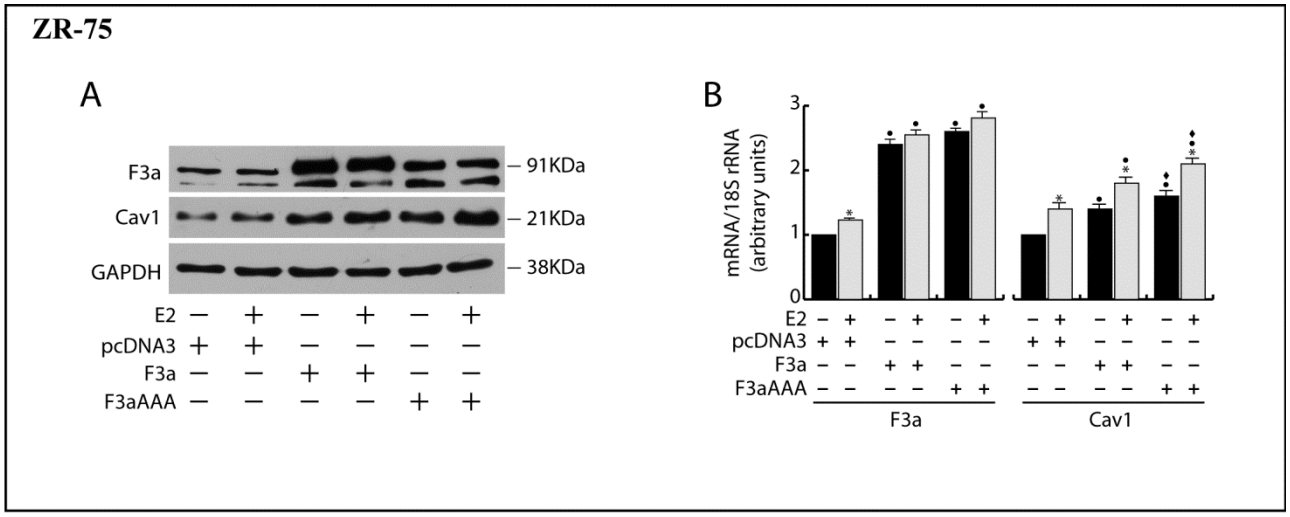


Figure 5. Cav1 expression depends on E2 and FoxO3a in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells were either transiently transfected with F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a, serum starved after 5 h and treated the next day with 100 nM E2 for 24 h. Cells were then harvested and total proteins and RNA were extracted, and subjected to WB (**A and C**) and RT-PCR analysis (**B and D**), respectively, for F3a and Cav1 expression assessment. (**E**) MCF-7 cells were seeded in growing medium, serum starved the next day for 24 h, pre-treated or not for 1 h with the pure antiestrogen ICI 182.780 and then treated with increasing concentrations of E2 (0, 1, 10, and 100 nM). (**F**) MDA-MB-231 cells were transiently transfected with F3a or pcDNA3 as control, serum starved for 24 h and then treated or not with 100 nM E2. After 24 h of E2 treatment, total proteins were extracted and subjected to WB analysis. GAPDH was analyzed as loading control in WB assays. For RT-PCR assays, each sample was normalized to its 18S rRNA content.Results are reported as the mean ±s.d. of at least 3 independent experiment. *, $P < 0.01$ vs. untreated; ●, $P < 0.01$ vs. pcDNA3; ♦, $P < 0.01$ vs. F3a; □ , $P < 0.05$ vs. siScramble.



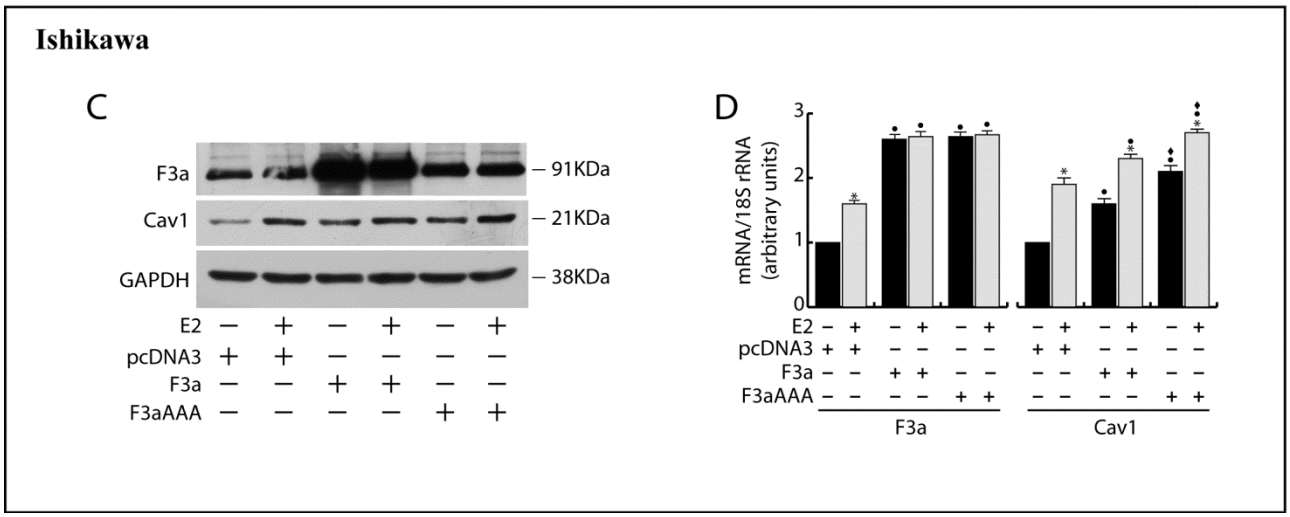


Figure 6 FoxO3a induces Cav-1 expression in ERα+cancer cells.
Double sets of ZR75 and Ishikawa cells were transiently transfected with F3a, F3aAAA or pcDNA3, as described in Materials and Methods. Five hours after transfection, cells were starved for 24h and then treated or not with 100nM E2 for additional 24h. Total proteins (**A and C**) and RNA (**B and D**) were extracted and subjected to WB and RT-PCR analysis respectively, to assess FoxO3a and Cav-1 expression. GAPDH was used as loading control in WB analysis.In RT-PCR assays each sample was normalized to its 18S rRNA content. Results are the mean ±s.d. of at least three independent experiments. *, $P < 0.01$ vs untreated; ●, $P < 0.01$ vs pcDNA3; ♦, $P < 0.01$ vs corresponding F3a.

Cav1 is a mediator of FoxO3a-dependent inhibition of migration, invasion, and growth in suspension in ERα+ breast cancer cells

Cav1 involvement in FoxO3a-mediated inhibition of motility, invasiveness, and colonies formation was assessed by silencing experiments using specific siRNAs against Cav1 (siCav1) in ERα+ breast cancer cells, (***Fig. 7A–D***). Cav1 silencing was able to counteract FoxO3a effects, leading to an overall increase of cell migration and invasion in MCF-7 cells, although F3a and F3aAAA overexpression did not contribute to such increase, nor was siCav1 sufficient to completely reverse the inhibitory effect exerted by E2 treatment (***Fig. 7A and B***). A similar trend was observed in soft agar experiments, where the number of colonies was much greater in siCav1 samples, especially under E2 treatment (note that ERα protein content was not affected by siCav1, ***Fig. 7D***), compared with the respective controls (siScramble) (***Fig. 7C***). Again, F3a and F3aAAA did not have any additive effect on colony growth (***Fig. 7C***).

These results show how, in MCF-7, FoxO3a control of cell migration, invasion, and anchorage-independent cell growth depends, in part, on Cav1, while it is strictly linked to ERα expression (***Fig. 3***). Indeed, in Cav1-negative T47D cells, which, in addition, bear a very low content of ERα, F3a, and F3aAAA overexpression did not lead to any significant decrease in motility, invading potential and colony formation in soft agar, reflecting a sort of compromise between the results observed following either Cav1 or ERα silencing in MCF-7 cells (***Figs. 3 and 7E–G***), thus indicating that these 2 proteins are mediators of both E2 and FoxO3a activity.

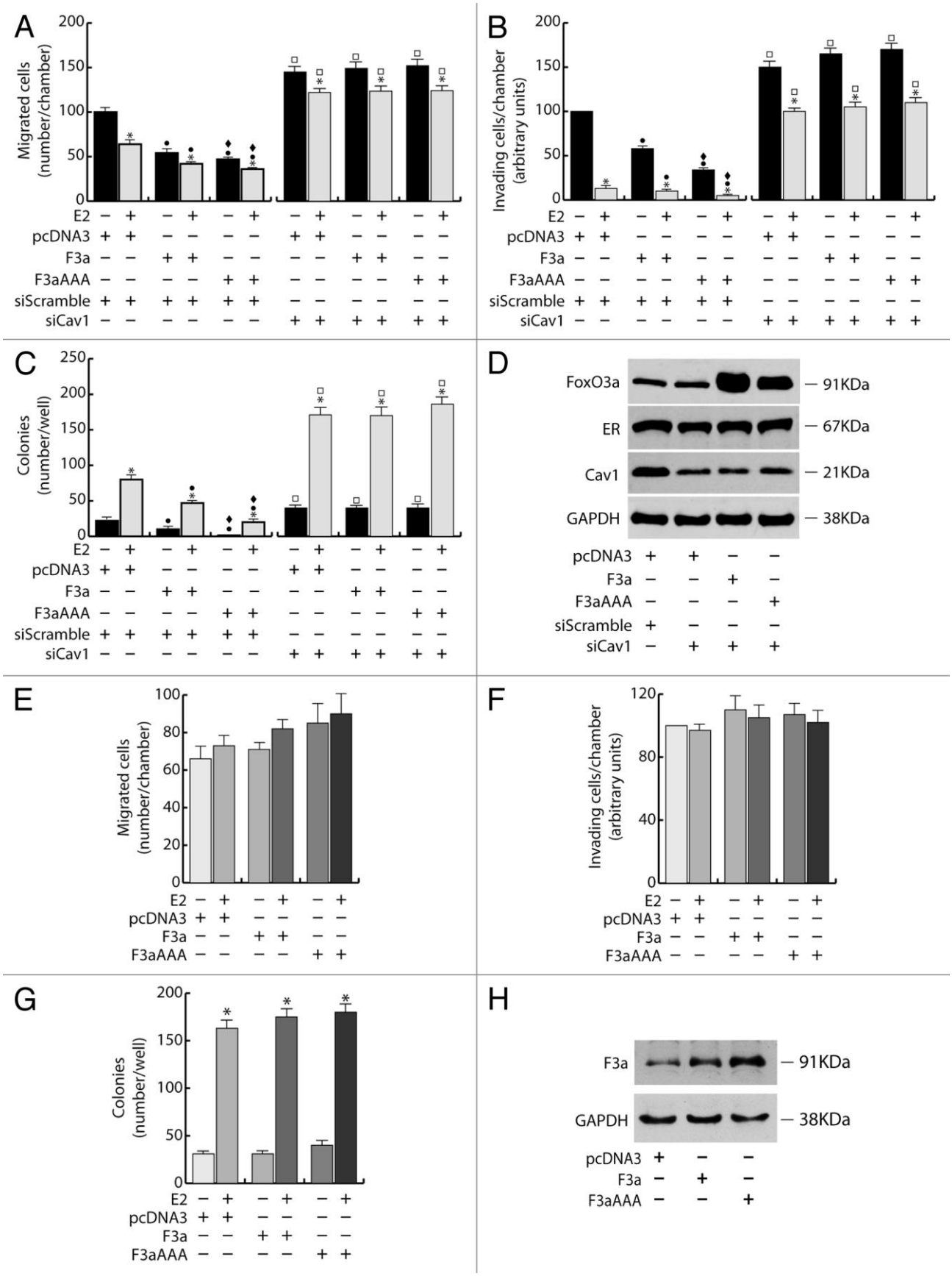


Figure 7. Cav1 is a mediator of FoxO3a dependent inhibition of migration, invasion and growth in suspension of ERα+ breast cancer cells. (**A–D**) Two double sets of MCF-7 cells were silenced for Caveolin-1 (siCav1), using siScramble as control. After 5 h cells were switched to PRF-SFM and transiently transfected with F3a, F3aAAA, or pcDNA3. Next day cells were harvested and one set of each experiment was subjected to

migration, invasion, and soft agar assay, in the presence or in the absence of E2. Migrated (**A**) and invading (**B**) cells were evaluated after 24 h and 72 h of incubation,
respectively. In soft agar assay, colonies ≥50 μm diameter formed after 14 d from plating were counted under the microscope (**C**). Transfection efficiency was evaluated by WB analysis on total protein extracted by the second set of cells; GAPDH was used as loading control (**D**). Results are the mean ±s.d. of at least 3 independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦, $P < 0.01$ vs. corresponding F3a; □ , $P < 0.01$ vs. corresponding siScramble. (**E–H**) A double set of T47D cells were transiently transfected with F3a, F3aAAA or pcDNA3. After 5h cells were switched to PRF-SFM and the next day one set of cells was harvested and subjected to migration (**E**), invasion (**F**), or soft agar assay (**G**), with or without 100 nM E2.
Migrated and invading cells were counted after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies formed after 14 d from plating were exposed to MTT and counted under the microscope. The second set of cells was lysed, and total protein was used for WB analysis to assess transfections efficiency; GAPDH was used as loading control (**H**). Results are the mean ± s.d. of at least 3 independent experiments. *, $P < 0.01$ vs. untreated.

FoxO3a binds to and trans-activates the Cav1 promoter in MCF-7 cells

To deepen the understanding of the mechanism underlying the FoxO3a/ERα interplay in Cav1 induction, through an accurate analysis of the Cav1 promoter (GenBank accession #AF095591.1), we verified the presence of several Forkhead core sequences (FKHE), and we questioned if any of the identified regions may be involved in the FoxO3a/ERα-mediated regulation of Cav1 gene expression in ERα+ breast cancer cells.

To this aim, a vector bearing the luciferase gene under the control of the -837/-36 region of Cav1 promoter (pGL3-cavFL) was co-transfected with F3a or F3aAAA in MCF-7 cells and exposed or not to E2 treatment. In line with the results reported in ***Figure 5A and B***, E2 stimulation significantly induced the Cav1 promoter activity, and such effect was increasingly higher in F3a- and F3aAAA-transfected cells (***Fig. 8A***). Interestingly, the construct pGL3/SRE1/2 (nt −837/−355), although containing FKHE core sequences, failed to be induced by FoxO3a but still weakly responded to hormone stimulation, most likely for the presence of Sp1 and AP-1 sites; on the contrary, the construct pGL3/SRE3 (nt −354/−36), bearing only one FKHE motif (nt −305/−299) and several Sp1 and AP-1 sites, was induced by both E2 and overexpressed FoxO3a, with a trend comparable to that observed with the pGL3-cavFL construct (***Fig. 8A***).

The involvement of E2 and FoxO3a in the transcriptional activation of the Cav1 promoter was corroborated by chromatin immunoprecipitation (ChIP)

experiments, which evidenced a significant recruitment of FoxO3a on the region containing the −305/−299 FKHE sequence. Once again, E2 treatment strongly increased FoxO3a occupancy of the promoter, especially in F3a- and F3aAAA-overexpressing samples (***Fig. 8B***). A similar pattern was observed in Polymerase II (PolII) precipitates, confirming that E2 and FoxO3a, both independently and synergistically, are able to induce Cav1 gene transcription (***Fig. 8B***).

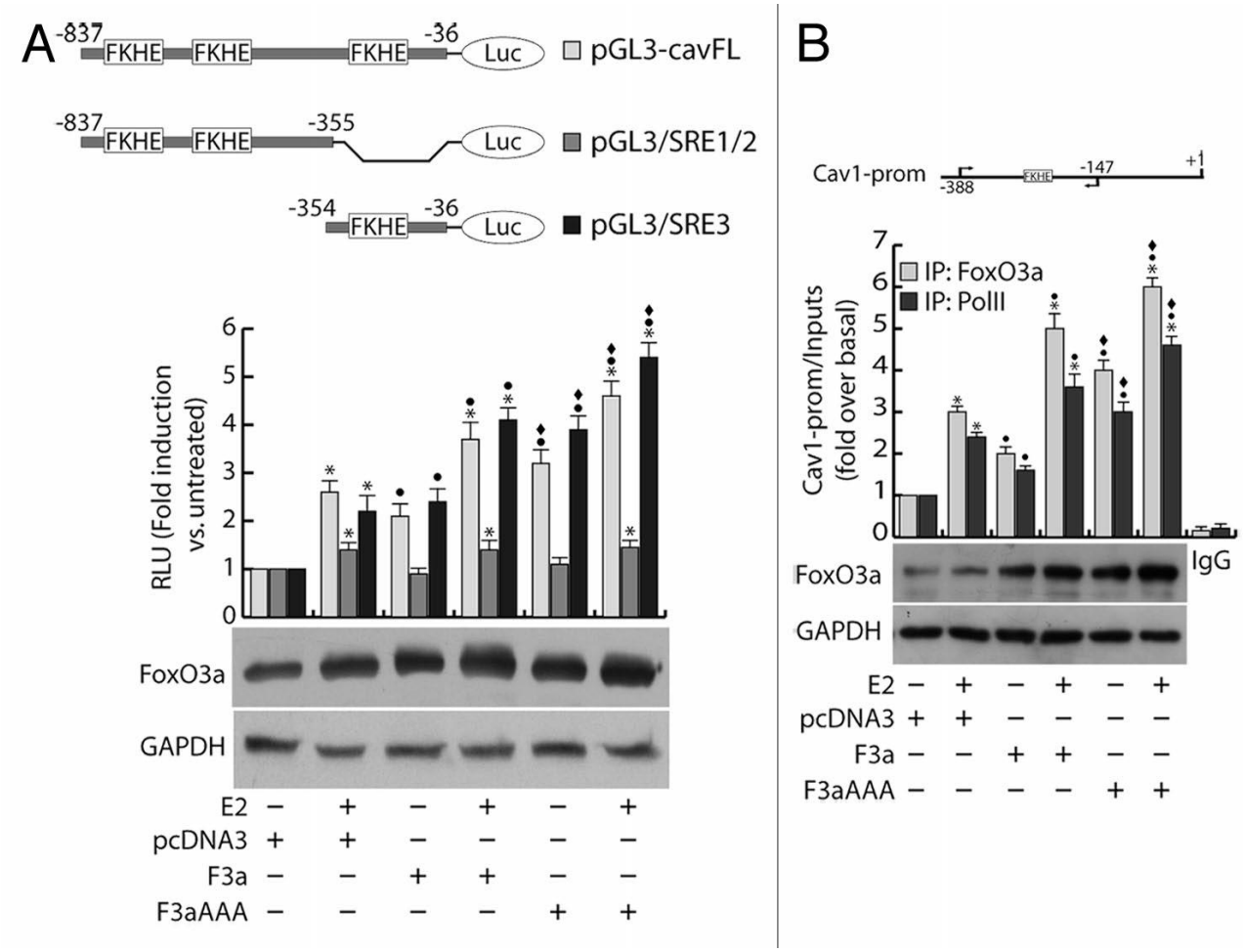


Figure 8. FoxO3a binds to and transactivates the Cav1 promoter. (**A**) MCF-7 were seeded in culture medium on 24-well plates, serum starved for 24 h, co-transfected in PRF-CT with pGL3-cavFL, or pGL3/SRE1/2, or pGL3/SRE3 and pRL-Tk, in presence of either pcDNA3 or F3a or F3aAAA vectors. After 6 h, E2 (100 nM) was added to the medium, where opportune, and the next day cells were harvested, and luciferase activity was evaluated. Cell extracts were also processed by WB analysis to assess F3a and F3aAAA transfection efficiency; GAPDH was used as loading control. (**B**) ChIP analysis was performed on the nuclear extracts from subconfluent MCF-7 cells seeded in 15 cm dish diameter, switched to PRF-SFM, and transfected with pcDNA3, F3a, or F3aAAA vectors. Twenty-four hours after transfection, the cells were treated with 100 nM E2 for 30 min or left untreated. The FKHE-containing Cav1 promoter region, precipitated with either anti-FoxO3a or anti-PolII pAbs were amplified using a specific pair of primers reported in "Materials and Methods". E2-treated samples were also precipitated with normal rabbit IgG and used as negative control. FoxO3a expression in transfected samples was analyzed by WB on Cytosolic lysates from the same set of cells. Data represents the mean ±s.d. of 3 independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.05$ vs. corresponding pcDNA3; ♦, $P < 0.05$ vs. corresponding F3a.

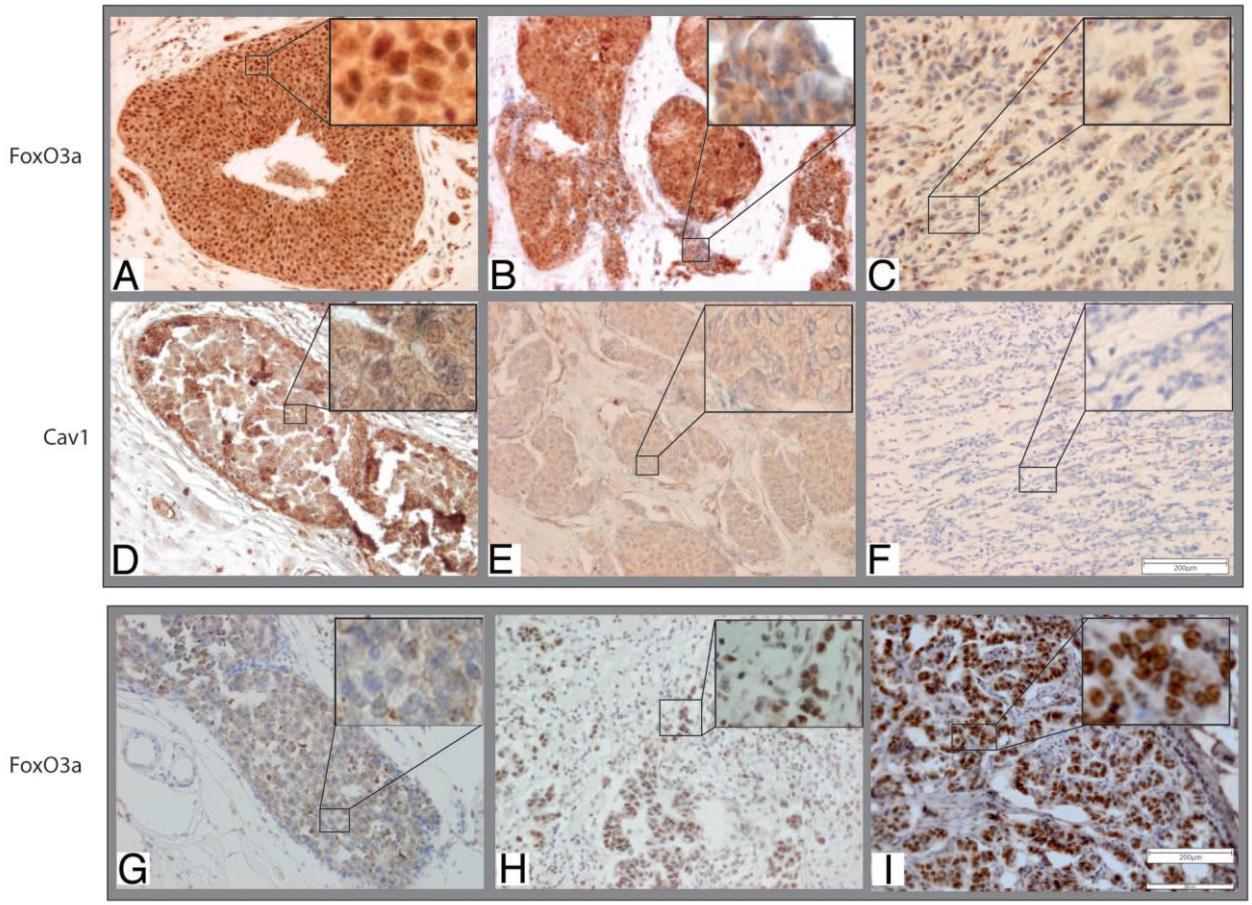
Nuclear FoxO3a correlates in an opposite way with the tumor grade and the invasive phenotype in ERα+ and ERα−breast tumors

Tissue specimens from ductal carcinomas in situ (DCIS) and invading ductal carcinomas (IDC) (***Fig. 9J***) were analyzed to investigate if FoxO3a expression could correlate with the tumor grade and the invasive potential in ERα+ and ERα− breast tumors, as well as with Cav1 expression (in ERα+ tumors only).

In all sections, tumor cells were clearly distinguishable from either infiltrating immune cells or stromal cells. In non-invading, well-differentiated ERα+ tumors, FoxO3a was strongly expressed, showing a very high nuclear localization (***Fig. 9A***).

Strikingly nuclear FoxO3a positivity was gradually lost in invading and less differentiated cells (see insets in ***Fig. 9B***), while cytoplasmic localization was not as indicative. Concomitantly, Cav1 expression tended to decrease from tumors with positive to negative FoxO3a nuclear staining, and was completely lost in highly invading ERα+ tumors (***Fig. 9D–F***). Statistical analysis of these samples showed that both FoxO3a nuclear expression and Cav1 were inversely correlated with tumor grade and the invasive potential, while cytosolic FoxO3a did not result to be significantly correlated with any clinicopathological feature (***Fig. 9K***); moreover, Cav1 expression resulted directly correlated with FoxO3a nuclear content (***Fig. 9K***).

On the contrary, a very weak or even absent FoxO3a nuclear localization was observed in intraductal, well delimited areas of ERα− tumors (***Fig. 9G***), while a very strong nuclear staining was detected in invading areas of the same samples (***Fig. 9H***) and in clearly invasive carcinomas (***Fig. 9I***). This observation was confirmed by statistical analysis that evidenced a direct correlation between FoxO3a expression and both tumor grading and the invasive potential of ERα− breast cancer tissues (***Fig. 9L***).



J

	Grading				
	G1	G2	G3	Invasive	Total
ER+	5	20	15	25	40
ER-	4	13	23	25	40

K

		FoxO 3a	Cav1
Invasion	r	-0,3535	-0, 4310
	p	0,047	0,0 138
Grading	r	-0, 4123	-0, 5887
	p	0,0 190	0,00 6
Cav1	r	0,6232	
	p	<0,0001	

L

		FoxO 3a
Invasion	r	0, 5805
	p	0,0073
Grading	r	0, 4730
	p	0,0352

Figure 9. Nuclear FoxO3a is highly expressed in non-invasive ERα+, and in invasive ERα− breast tumors. FoxO3a (**A–C**) and Cav1 (**D–F**) expression in ERα+ breast tumors and FoxO3a (**G–I**) in ERα− breast tumor samples. IHC was conducted on tissue sections deriving from biopsies diagnosed as DCIS (**A and D**), microinvasive DCIS (**B and E**), DCIS with contiguous IDC areas (**G and H**) and highly aggressive IDC (**C, F, and I**). Representative fields were photographed at 20× magnification. Insets, showing details of proteins subcellular localization, were taken at 100× magnification. (**J**) Samples descriptions and classification; (**K**) correlation between nuclear FoxO3a or Cav1 content and the tumor grading and invasive potential in ERα+ breast cancer samples; (**L**) correlation between nuclear FoxO3a content and the tumor grading and invasive potential in ERα− breast cancer samples. The correlation coefficient (*r*) and the statistical significance (*P*) are reported.

Discussion

FoxO transcription factors are crucial for regulating a myriad of physiological processes, including proliferation, metabolism, cell differentiation, cell cycle arrest, DNA repair, and apoptosis.

FoxOs also play important roles in tumorigenesis, since they have been shown to be deregulated in many types of human cancers, and restoring their expression/activity has been shown to be effective in tumor suppression *(Yang JY et al,2011).*

The involvement of FoxOs in tumor metastasis is controversial, e.g., FoxO3a has been reported to have either a protective or a promoting role on cell motility and invasion *(Belguise K et al, 2007; Storz P et al, 2009)*.Our hypothesis was that such a difference might be ascribed to ERα status, since activated FoxO3a was able to reverse the invasive phenotype of ERα+ breast cancer cells *(Belguise K et al, 2007)* while promoting tumor cell invasion in other cancer cell lines, which, notably, were all ERα− *(Storz P et al, 2009)*. Thus, the present study was aimed to verify if the effect exerted by FoxO3a on the metastatic potential of ERα+ breast cancer could derive from a general mechanism through which FoxO3a cooperates with the nuclear receptor in reducing motility and invasiveness of ERα+ tumors, while in absence of the receptor FoxO3a favors a more migrating and invasive phenotype. Indeed, since Erα signaling is well known to strongly correlate with a lower invasiveness and reduced motility of breast cancer cells *(Sisci D et al, 2010)* and considering that increasing evidences recognize Forkhead factors as important modulators of ERα transcriptional activity, *(Schuur ER et al, 2001; Zhao HH et al, 2001; Morelli C et al, 2010)* it won't surprise to ascertain that, in ERα+ tumors, FoxO3a could reduce cell migration and invasion through a functional interaction with ERα. On the other hand, in ERα− tumors, the absence of the receptor could enable FoxO3a to trigger some different pathway that leads to an opposite outcome.

To prove our hypothesis, minimally motile and invasive ERα+ MCF-7 and ZR-75 breast cancer cell lines have been transfected with wild-type F3a and constitutively active F3aAAA mutant, and the effects on cell migration, invasion, and colony formation in soft agar were observed. The results presented here show that FoxO3a overexpression reduces the migratory and invasive potential, as well as anchorage-independent growth (a hallmark of tumor progression), in ERα+ tested cells. It is worth noting that, in all experiments, the constitutively active mutant F3aAAA was always more effective than the wild-type FoxO3a, suggesting that the regulation of the above-mentioned features could occur at the transcriptional level, through the induction of Forkhead responsive genes. Moreover, the expected reduced motility and invasiveness of ERα+ cells upon E2 stimulation *(Sisci D et al, 2010)* was more evident in F3a and, especially, in F3aAAA-overexpressing cells, providing evidence that E2 and FoxO3a act synergistically on these 2 features (***Fig. 1A and B; Fig. 2, upper panels***). On the contrary, E2 stimulation does not show an anti-metastatic behavior in presence of growth factors, since it favors the anchorage-independent growth *(Manni A et al, 1991)*, suggesting that other growth factors regulated pathways do prevail on that of ERα in the control of this feature. However, in line with our previous observations *(Morelli C et al, 2010)*, FoxO3a overexpression was able to counteract the proliferative effect of E2, and its silencing led to an increase in basal as well as in E2-dependent cell growth (***Fig. 1C$_1$ and C$_2$***). Taken together, these results suggest, once again, that FoxO3a might act as a corepressor (e.g., by quenching E2/ERα dependent proliferative signals *(Morelli C et al, 2010)*) or a co-activator (e.g., by potentiating E2/ERα mediated inhibition of cell motility and invasion *(Sisci D et al, 2010)*) for ERα *(Zhao HH et al, 2001)*.

More importantly, ERα is the key regulator of FoxO3a function, as evidenced by the opposite behavior of overexpressed F3a (and F3aAAA) in ERα-silenced cells if compared with the corresponding ERα-expressing samples (***Fig.3***). Thus,

the lack of the hormone receptor is responsible for the switch of FoxO3a biological function, which shifts from inhibitory (when ERα is present) to stimulatory (when ERα is absent) on cell motility, invasion, and growth in suspension.

This is confirmed by the fact that FoxO3a overexpression exhibits a stimulating (rather than inhibitory as in ERα+ cells) effect on the same features in ERα− MDA-MB-231,MDA-MB-468, and MDA-MB-435S breast cancer cells.

Notably, since the results observed in ERα+ and ERα− breast cancer cells following F3a and F3aaAAA ectopic expression, were similar to those obtained in non-breast cancer Ishikawa (ERα+ human endometrial adenocarcinoma) and HeLa (ERα− human cervical cancer) cell lines, respectively, we could assume that FoxO3a controls cell migration, invasion, and growth in suspension with a general, not tissue-specific, mechanism, which seems to depend on ERα expression (***Fig. 4; Fig. 2***).

Our results also show how Cav1 represents the ultimate downstream target through which FoxO3a modulates the metastatic potential of ERα+ cells. Cav1 is a multifunctional scaffolding protein that is associated with cell surface caveolae and the regulation of lipid raft domains. Cav1 regulates multiple cancer associated processes, including cellular transformation, tumor growth, cell migration and metastasis, cell death and survival, multidrug resistance, and angiogenesis. In breast cancer, Cav1 seems to function as a tumor suppressor *(Sotgia F et al, 2006)*. In fact, Cav1 mRNA and protein are downregulated or absent in primary human cancers as well as in several mouse and human breast cancer cell lines. Forced re-expression of Cav1 in transformed mammary cell lines abrogates numerous of their tumorigenic properties, including anchorage-independent growth and invasiveness *(Fiucci G et al, 2002)* and suppresses growth of breast cancer cell-derived xenografts in nude mice *(Wu P et al, 2008)*. Moreover, $Cav1^{-/-}$ mice showed an accelerated onset of mammary tumors and lung metastases *(Williams TM et al, 2004)*. In accordance, Cav1 expression has

been inversely related to the grade of the primary breast tumors and its upregulation was found to reduce metastasis to distant organs *(Sloan EK et al, 2004)*.

In light of this evidence, we questioned if FoxO3a could exert a protective role in ERα+ breast cancer cells through the induction of Cav1 expression. Indeed, in all ERα+ cells tested, FoxO3a overexpression increased the RNA and protein amounts of Cav1, and such increase was additive to that observed under E2 treatment, suggesting that ERα is also involved in the transcriptional induction of Cav1 (***Fig. 5***), which, in turn, seems to be the effector of a less aggressive phenotype, as evidenced by Cav1-silencing experiments (***Fig. 7A–D***) and by the fact that F3a and F3aAAA overexpression failed to inhibit migration, invasion, and growth in suspension in Cav1-negative T47D cells, despite the presence of a low, but still functional, content of ERα (***Fig. 7E–H***).

Since the highest induction of Cav1 has always been observed in F3aAAA-transfected cells, Cav1 regulation by FoxO3a and estrogens at the transcriptional level was investigated. In fact, the 5′-flanking region of the *CAV1* gene, including the promoter region, bear several perfect and predicted forkhead consensus sequences, one of which (at position −1814, located above the promoter sequence) has been reported to be responsible for forkhead dependent *CAV1* gene regulation *(van den Heuvel AP et al, 2005)*. However, as the same authors stated, it is possible that other FKHE, also present within the 5′-flanking region, may play a role in Cav1 transcriptional activation by FoxO as well. Indeed, the data presented here clearly show how FoxO3a is able to induce Cav1 transcription by binding to a FKHE motif, mapping nt −305/−299 of its promoter;in addition, the FoxO3a-dependent Pol II recruitment confirms the occurrence of a transcriptional event (***Fig. 8***). To explain the induction exerted by E2, alone or in combination with FoxO3a, on Cav1 expression, we exclude, at the present stage, the direct involvement of ERα in the transcriptional process, since an integrated analysis of ERα binding sites upstream of the Cav1 gene,

through Myles Brown lab data sets (http://research.dfci.harvard.edu/brownlab/datasets/index.php?dir=ER_whole_human_genome/) *(Carroll JS et al, 2006)* and Cistrome-web application (http://cistrome.dfci.harvard.edu/ap/), evidenced that ERα recruitment to the chromatin occurs at a very large distance from the promoter, on 3 distinct positions around 80–100 Kb upstream of the transcription start site. No ERα binding is reported in the data sets at the promoter level or in its close proximity, as also confirmed by ChIP experiments conducted on several predicted estrogenresponsive motifs identified within the +1/−5000bp region (data not shown). Additionally, neither Sp1 nor AP-1 transcription factors, 2 well-established mediators of the ERα "non-classical" genomic pathway *(Safe S et al, 2008)* that have been reported to transcriptionally cooperate with FoxO3a, *(Lützner N et al, 2012; Luo X et al, 2007)* resulted to be involved in Cav1 regulation.

In fact, both Sp1 silencing and c-Jun inhibition achieved through the dominant-negative (DN)/c-fos plasmid *(Ahn S et al, 1998)* did not lead to any significant decrease in FoxO3a/E2-dependent Cav1 promoter activation, nor to a reduction of Cav1 protein content (data not shown). Despite these observations, the evidence that liganded ERα induces Cav1 expression, and that E2 and FoxO3a, separately or synergistically, lead to a significant increase of Pol II recruitment on the Cav1 promoter region (***Fig. 8***), suggests that it would be interesting to investigate, by means of the recent and fascinating techniques Chromosome conformation capture (4C) technology and detection of loops in DNA-picked chromatin (DPC), *(Simonis M et al, 2007; Abbondanza C et al, 2011)* if the combined effect of E2 and FoxO3a on Cav1 expression could be ascribed to the interaction of at least one of the 3 above mentioned ERα binding sites, at 80–100 Kb upstream of the transcription start site, where FoxO3a is recruited to the *CAV1* gene promoter (ongoing experiments). In fact, recent studies using tiled microarrays to identify the ERα interacting sites of estrogen responsive genes,

showed that EREs can function as enhancer elements far away (up to 100 Kb) from gene promoters, and that other cooperating transcription factors (e.g., FoxA1, AP1 and Sp1) can participate with ERα to regulate the expression of E2-induced genes (Carroll JS et al, 2005; Carroll JS et al, 2006).

Taken together, the results obtained in ERα+ cancer cells show that FoxO3a-dependent decrease of migration, invasion, and colony formation is mediated by both ERα and Cav1, as confirmed by knockout experiments of these two factors (***Figs. 3, 5, and 7***). In particular, ERα cooperates with FoxO3a in the transcriptional induction of Cav1, which, in turn, is responsible of the reduced aggressive phenotype of FoxO3- overexpressing ERα+ cells (***Fig. 10***).

On the other hand, several reports called into question Cav1 role as a tumor suppressor, since it has been found overexpressed in highly aggressive inflammatory breast cancer (IBC) human specimens and cell lines *(Van den Eynden GG et al, 2006)* as well as in invasive human breast cancers samples, where its expression was significantly associated with basal-like phenotype, high histological grade, shorter disease-free and overall survival, and, more interestingly, lack of steroid hormone receptors positivity*(Savage K et al,2007; Elsheikh SE et al, 2008)*. Moreover, in ERα− cancer cells, Cav1 has been found in membrane protrusions, where it promotes tumor cell migration and invasion by regulating either the function of membrane type 1 matrix metalloproteinase (MT1-MMP) *(Yamaguchi H et al, 2009)*, or, when phosphorylated (pY14Cav1), the focal adhesion turnover (Joshi B et al, 2008). Therefore, we investigated if the more aggressive phenotype of FoxO3a overexpressing ERα− cells could depend, also in this case, on Cav1 induction. However, no differences in Cav1 levels or phosphorylation status have been detected in ERα− cells following FoxO3a overexpression, nor E2 treatment, possibly through ERβ, has been able to induce Cav1 expression (***Fig. 5***, and data not shown).

Although MMP-9 and MMP-13 induction has been proposed as the mechanism through which FoxO3a increases invasion of cells lacking the hormone receptor,

(Storz P et al, 2009) not all the ERα− cell lines tested do express these MMPs, or do express negligible levels.

Moreover we failed to detect a reproducible increase in MMP-9 transcripts and in MMP-13 mRNA and protein in FoxO3aoverexpressing cells (data not shown), thus other markers are currently being investigated in our laboratory to justify the higher motility and greater invading ability induced by FoxO3a in ERα− cells. However, it is worth to underline that ERα silencing is a sufficient condition to reverse the effect of FoxO3a on migration, invasion and colony formation in ERα+ cells (***Fig. 3***), thus ERα seems to be a pivotal regulator of FoxO3a function, which switches from protective to malignant depending, respectively, on the presence or absence of the hormone receptor. A schematic representation of our findings is shown in ***Figure 10***.

Finally, an immunohistochemical study from Yoshino's research group showed that nuclear FoxO3a associates with IDC and lymph node metastasis, and the same authors speculated that, in some cases, aberrant activation of FoxO3a may cause the recruitment of metastasis-related molecules, instead of inducing apoptotic genes *(Jin GS et al, 2004)*. Since no association with ERα status has been considered in this study, it might be possible that nuclear FoxO3a could correlate to a more metastatic phenotype only in the subset of ERα− IDC. In line with this hypothesis, nuclear FoxO3a has been recently proposed as a good prognostic factor in luminal- like breast cancer, which contain principally ERα+ cases, *(Bertos NR et al, 2011)* where it directly correlates with biomarkers of good prognosis and inversely with mitotic counts and tumor grade. Moreover, with respect to patient outcome, FoxO3a nuclear localization was associated with longer breast cancer specific survival and longer distant metastasis-free interval, independently of the well-established breast cancer prognostic factors *(Habashy HO et al, 2011)*.

The screening of nuclear FoxO3a on opportunely selected ERα+ and ERα− tissue samples from patients with breast cancer of ductal origin gave results that

perfectly fit with the above-mentioned reports and also confirm the in vitro studies presented in this work. Moreover, the co-expression of Cav1 and FoxO3a in ERα+ tumors, together with the functional link provided by our in vitro data, supports a potentially important role for these 2 proteins in predicting a better tumor prognosis. However, a more systematic evaluation within various subtypes of ERα+ and ERα− non-invasive and invasive breast cancers, in absence or in presence of lymph node and/or long distance metastasis, would help to better clarify the biological and prognostic role of FoxO3a protein expression, also with respect to its subcellular localization.

For instance, since no correlation has been found between FoxO3a and ERα *(Habashy HO et al, 2011)*, the loss of an active (nuclear) FoxO3a might be predictive of a worse phenotype in the subset of ERα+ breast cancers that do not respond to therapy. At the same time, a more accurate immunohistochemical analysis on the biological link between FoxO3a and Cav1 in hormone-positive tumors needs to be addressed. In fact, although Cav1 expression has been associated with lack of the steroid hormone receptor *(Elsheikh SE et al, 2008)*, its positivity in luminal-like tumors could represent a good prognostic factor when associated to a FoxO3a nuclear prevalence.

In conclusion, the results presented here give new insights on the functional role of nuclear FoxO3a, whose overexpression seems to be associated to a low motile phenotype in ERα+ breast cancers and to a more metastatic potential in those lacking the hormone receptor, harboring the idea that ERα may represent the molecular switch determining FoxO3a biological behavior.

These evidences clearly suggest that FoxO3a has the potential to become a relevant prognostic factor and a suitable pharmacological target to be exploited in combination therapies for both ERα+ (through FoxO3a activation) and ERα− (through FoxO3a disruption) breast cancer patients.

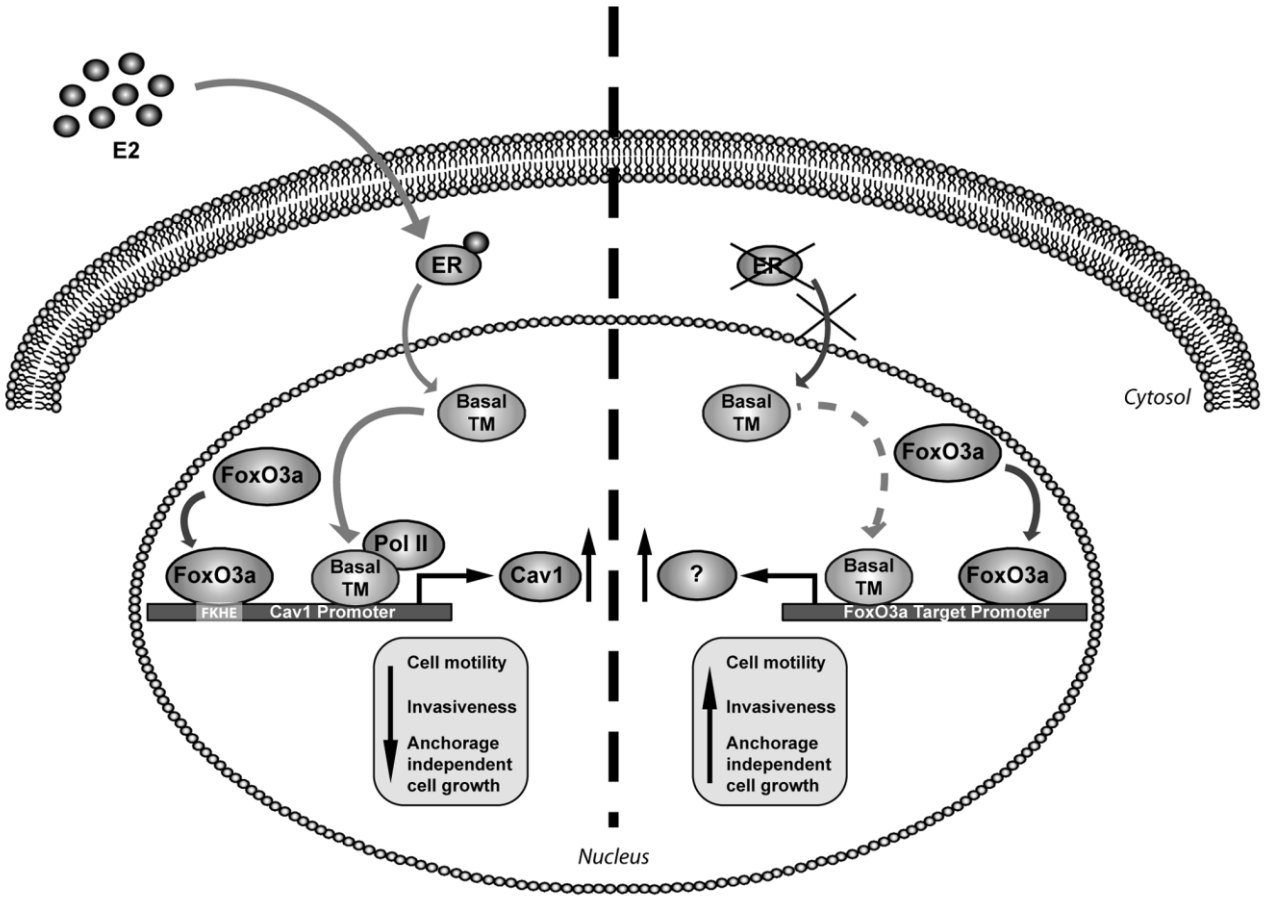


Figure 10. Proposed model for FoxO3a-mediated control of cell motility and invasiveness in presence or absence of ERα. F3a and ERα synergistically induce the expression of Cav1, which, in turn, reduces cell motility and invasiveness of ERα+ breast cancer cells. Transcriptionally active F3a binds to a FKHE located on the Cav1 proximal promoter and increases the recruitment of RNA Polymerase II, which is enhanced upon E2 stimulation. The lack of the hormone receptor enables active F3a to behave in an opposite fashion, thus increasing cell motility and invasion. Basal TM, basal transcriptional machinery.

Materials and Methods

Cell culture, conditions, and treatments

The human breast cancer epithelial cell lines MCF-7, ZR75, T47D, MDA-MB-231, and MDA-MB-468 and the cervical epithelial cell line, HeLa, were purchased from Interlab Cell Line Collection, ICLC, Italy. Ishikawa human endometrial cancer cell line was obtained from D Picard (University of Geneva). MCF-7 and ZR75 were maintained in DMEM/Ham F-12 medium (1:1) (DMEM/F-12) supplemented with 5% FBS. Ishikawa and HeLa cells were grown in MEM containing 10% FBS and 1% nonessential amino acids. MDA-MB-231 and MDA-MB-468 cells were cultured in 10% FBS DMEM. T47D cells were cultured in RPMI containing 10% FBS, 2.5 g/ml glucose, 1% Na-Pyruvate, 10 nM Hepes, and 0.2 U/ml insulin. Additionally, culture media were supplemented with 100 IU/ml penicillin, 100 ng/ml streptomycin, and 0.2 mM L-glutamine. For experimental purposes, cells were synchronized in phenol red-free and serum-free media (PRF-SFM) for 24 h and then, where opportune, switched to PRF-media containing 5% charcoal-treated FBS (PRF-CT) or FBS (ERα+ and ERα− cells, respectively), in presence or absence of 17β-estradiol (E2, Sigma-Aldrich). All media and reagents were purchased from Invitrogen.

Plasmids and transfections assays

The following plasmids were used: pcDNA3 empty vector (Invitrogen); 1038 pcDNA3 flag FKHRL1 (F3a) encoding full-length FoxO3a and 1319 pcDNA3 flag FKHRL1 AAA (F3aAAA), encoding the constitutively active triple mutant of FoxO3a (provided by William Sellers, Addgene plasmids 10708 and 10709, *(Ramaswamy S et al, 2002)* respectively). MCF-7, ZR75, and MDA-MB-231 and MDA-MB-468 cells were resuspended in PRF-growing medium (PRF-GM) and transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, while transfection of T47D, Ishikawa and HeLa cells were conducted with FuGENE HD (Promega). Six hours after

transfections, cells were synchronized for 24 h and then subjected either to migration, invasion, and soft agar assays or switched to FBS (ERα− cells) or PRF-CT, in presence or absence of E2 (ERα+ cells), for protein and RNA extraction purposes.

For luciferase assays, the following constructs of the Cav1 promoter *(Cao S et al, 2005)* were used: pGL3-cavFL, driving the expression of firefly luciferase under the control of the Cav1 promoter full-length (nt −837/−36 from the ATG), pGL3/SRE1/2 (nt −837/−355) and pGL3/SRE3 (nt −354/−36). Transfections were performed using FuGENE HD. Luciferase activity was measured using the dual-luciferase assay system, normalized to pRL-Tk activity (both from Promega), and expressed as fold-induction over the control.

siRNA-mediated RNA interference

Custom-synthesized siRNA-annealed duplexes (25 bp double-stranded RNA [dsRNA]) were used for effective depletion of FoxO3a (siF3a) and Caveolin-1 (siCav1) transcripts. A scramble siRNA (siScramble) lacking identity with known gene targets was used as a negative control. Cells were transfected in suspension with Lipofectamine 2000 in PRF-GM, using the appropriate amounts of siRNA duplexes (Life Technologies). ERα silencing was conducted according to manufacturer's instructions using siER and the appropriate transfection reagent HiPerFect HTS Reagent purchased from Qiagen. For each silenced gene, at least 2 different siRNAs have been employed with comparable outcome.

Migration and invasion assays

Migration assays were performed as previously described *(Sisci D et al, 2010)*. Briefly, 6 h after transfection or silencing, cells were serum starved for 24 h, resuspended in PRF-SFM, and seeded (104 cells/insert) on the upper face of 24-well modified Boyden chambers (8 μm) (Corning); 500 μl of 5% PRF-CT with

or without 100 nM E2 (for ERα+ cells) or PRF-GM (for ERα− cells) were added to the bottom of the wells. After opportune incubation, migrated cells were stained with Coomassie brilliant blue and counted under the microscope. For invasion experiments, 30 µl of MatrigelÔ Basement Membrane Matrix (BD Biosciences) (1:3 dilution in PRFSFM) were coated on the internal surfaces of the Boyden chambers and let solidify at RT for 30 min. The lower chambers were loaded as described for migration assays. Cells suspended in 200 µl of 1% PRF-CT (ERα+ cells) or 1% FBS (ERα−cells), respectively, were plated into the upper chambers (105cells/insert). After the appropriate times of incubation, cells in the upper chamber were removed by a cotton tip; membranes were then mixed in methanol for 10 min at −20 °C, rinsed with PBS, stained with DAPI (Sigma Aldrich, Italy) for 5 min, rinsed again in PBS and dried. The filters were then detached from the chamber, and mounted onto slides using Fluoromount mounting medium (Sigma Aldrich) and observed under a fluorescence microscope (Olympus BX51 fluorescence microscope, Olympus Italia srl). Invading cells were photographed at 10× magnification using ViewFinderÔ Software, through an Olympus camera system dp50 and then counted using ImageJ software (NIH).

Anchorage-independent growth assay

Transfected or silenced ERα+ cells were seeded in 1 mL of 0.3% GellyPhor^{Ò} HR agarose (Euroclone S.p.A.) on top a base of 0.6% agarose in 12-multiwell plates in PRF-CT (2 × 104 cells/well) and treated with 100nM E2 or left untreated; ERα− cells were seeded in PRF-GM (3 × 104 cells/well). On day 14, the colonies (>50 µm) were exposed to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h, photographed at 4× magnification and counted under the microscope (Olympus BX51 microscope).

RNA extraction, reverse transcription, and real-time (RT)-PCR

Total RNA was isolated using TRI-reagent (Ambion) and treated with DNase I (Life Technologies). Two micrograms of total RNA were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was diluted 1:3 in nucleasefree water, and 5 µl were analyzed in triplicate by RT-PCR in a iCycler iQ Detection System (Bio-Rad) using SYBR green Universal PCR Master Mix (Bio-Rad) and the following pairs of primers: FoxO3a forward 5′-CAAACCCAGG GCGCTCTT-3′ and reverse 5′- CTCACTCAAG CCCATGTTGC T-3′ (68 bp); Cav1 forward 5′- CAGTTTTCAT CCAGCCACGG-3′ and reverse 5′- CGGATGGGAA CGGTGTAGAG-3′ (82 bp). Negative controls contained water instead of first-strand cDNA. Each sample was normalized on its 18S rRNA content. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. The final results were expressed as *n*-fold differences in gene expression relative to 18S rRNA and the calibrator, calculated using the $\Delta\Delta C_T$ method as follows:

n-fold = $2^{-(\Delta C_T \text{sample} - \Delta C_T \text{calibrator})}$, where the ΔC_T values of the sample and calibrator were determined by subtracting the average C_T value of the 18S rRNA reference gene from the average C_T value of the different genes analyzed.

Western blotting (WB) assays

Protein expression was assessed by WB assay as previously described *(Lanzino M et al, 2010)*. Total lysates were extracted using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate) plus inhibitors (0.1 mmol/liter Na3VO4, 1% PMSF, and 20 mg/ml aprotinin). The protein content was determined using Bradford dye reagent (Bio-Rad). Fifty µg of lysates were separated on an 11% polyacrylamide denaturing gel and transferred to nitrocellulose membranes. Proteins of interest were detected with specific

polyclonal (p) or monoclonal (m) antibodies (Abs), recognized by peroxidase-coupled secondary Abs, and developed using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech). The following Abs were used: anti-FoxO3a (75D8) pAb (Cell Signaling), anti-Cav1 (N-20) pAb, anti-ERα (F-10) mAb, and anti-GAPDH (FL-335) pAb (Santa Cruz Biotechnology). Images were acquired by using an Epson Perfection scanner (Epson).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as previously described *(Morelli C et al, 2010)*. The immuno-cleared chromatin was precipitated with anti-FoxO3a pAb (Abcam, USA) and anti-Polymerase II (N-20) pAb (Santa Cruz Biotechnology). Normal rabbit IgG (Santa Cruz Biotechnology) was used instead of primary Abs as negative controls. Immunoprecipitated DNA was analyzed by RT-PCR, as described above. A pair of primers (5′-GAGATGATGCACTGCGAAAA-3′ and reverse 5′-GCCAAAGGTTTGTTCTGCTC -3′) (242 bp) mapping the FKHE-containing Cav1 promoter region forward was used.

Tissue collection, immunohistochemistry (IHC), and data analysis

Formalin-fixed paraffin-embedded tissue sections were prepared from primary operable breast cancer cases (15 DCIS and 25 IDC from ERα+ tumors and an equal number from ERα−tumors) from patients under age 80 who underwent mastectomy at the Cosenza Hospital (Cosenza Hospital Authority) between 2011 and 2012. FoxO3a, ERα and Cav1 expression were assessed by IHC. The rabbit anti-FoxO3a pAb (cat. PA1-14171, Thermo Scientific) and the rabbit anti-Caveolin-1 pAb (N-20) (sc-894, Santa Cruz Biotechnology) were optimized at a working dilution of 1:200 in Dako Real antibody diluent (DAKO); the mouse anti-ERα (Clone 1D5, DAKO) was ready to use. Deparaffinization, rehydration, and antigen unmasking was obtained by incubation in tris-phospahte buffer (Envision Flex target retrieval solution) in a Pre-Treatment Module for Tissue

Specimens (PTLINK), according to the manufacturer's instructions (DAKO). The staining was performed in a Dako Autostainer Link48 immunostainer, using a linked streptavidin biotin technique (Envision Flex kit High pH, DAKO) in accordance with the manufacturer's instructions. Sections were counterstained in hematoxylin and coverslipped using DPX mounting medium (both from Sigma-Aldrich).

The expression and subcellular localization of FoxO3a and Cav1 were evaluated microscopically. Pictures of representative fields were taken at opportune magnification using ViewFinderÔ Software, through an Olympus camera system dp50.

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Red wine consumption may affect sperm biology: the effects of different concentrations of the phytoestrogen myricetin on human male gamete function. Aquila S, Santoro M, De Amicis F, Guido C, Bonofiglio D, Lanzino M, **Cesario MG**, Perrotta I, Sisci D, Morelli C.Mol Reprod Dev. 2013 Feb;80(2):155-65. doi: 10.1002/mrd.22145. Epub 2013 Jan 30.

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RESEARCH ARTICLE

Molecular Reproduction & Development 80:155–165 (2013)

Red Wine Consumption May Affect Sperm Biology: The Effects of Different Concentrations of the Phytoestrogen Myricetin on Human Male Gamete Function

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SUMMARY

Myricetin is a natural flavonoid, particularly enriched in red wines, whose occurrence is widespread among plants. Despite extensive research, the beneficial effects of Myricetin on human health are still controversial. Here, we tested the estrogen-like effect of the phytoestrogen Myricetin on human ejaculated sperm biology. To this aim, human normozoospermic samples were exposed to increasing concentrations (10 nM, 100 nM, and 1 μM) of Myricetin. Motility, viability, capacitation-associated biochemical changes (i.e., cholesterol efflux and tyrosine phosphorylation), acrosin activity, as well as glucose utilization and fatty-acid oxidation (i.e., glucose and lipid metabolism) were all significantly increased by low doses of Myricetin. Importantly, both estrogen receptors α and β (ERs) and phosphatidylinositol-3-OH kinase (PI3K)/AKT signaling are activated in the presence of Myricetin since these were both abrogated by specific inhibitors of each pathway. Our results show how Myricetin, through ERs and PI3K/AKT signalings, potentiates sperm function. This effect is dose-dependent at low concentrations of Myricetin (up to 100 nM), whereas higher amounts do not seem to improve any further sperm motility, viability, or other tested features, and, in some cases, they reduced or even abrogated the efficacy exerted by lower doses. Further studies are needed to elucidate if high levels of Myricetin, which could be attained even with moderate wine consumption, could synergize with endogenous estrogens in the female reproductive tract, interfering with the physiological sperm fertilization process.

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INTRODUCTION

Estrogens are steroid hormones that play a variety of physiological roles, particularly in development and maintenance of normal female sexual and reproductive function (Lanzino et al., 2008) and in male reproductive physiology

Abbreviations: Ab-ER, anti-ER antibody inhibition; BSA, bovine serum albumin; E2, 17-β-estradiol; ER[α/β], estrogen receptor [alpha/beta]; G6PDH, glucose-6-phosphate dehydrogenase; ICI, ICI 182.780 (ER inhibitor); LY, LY294002 (PI3K inhibitor); PI3K, phosphoinositol 3-kinase.

(Korach et al., 1996; Hess et al., 1997; Luconi et al., 2002; Ded et al., 2010; Sebkova et al., 2012). Estrogens signaling is mediated by two nuclear receptor, the estrogen receptor α (ERα) and β (ERβ), that function both as signal transducers and transcriptional factors that modulate expression of target genes (Heldring et al., 2007). Both receptors are present throughout the male reproductive tract as well as in germ cells (Adeoya-Osiguwa et al., 2003). In human spermatozoa, both ERs are located in the midpiece, while ERβ is also present in the tail (Aquila et al., 2004). Moreover, sperm possess the P450 aromatase responsible for the conversion of androgens to estrogens, which has been identified in rat sperm cytoplasmic droplets (Hess, 2000) and human sperm flagella (Aquila et al., 2002), thereby endowing them with the potential to synthesize estrogens, consistent with the similar aromatase activity detected in both Leydig and Sertoli cells (Carreau et al., 1999). The development of male transgenic mice lacking ERs (Eddy et al., 1996) or the aromatase enzyme (Robertson et al., 1999), as well as the discovery of mutations in both the human ERα (Smith et al., 1994) and aromatase (Carani et al., 1997) genes, have reinforced the idea that estrogens play a key role in the human male reproductive system.

Sperm are also exposed to estrogens in the female reproductive tract (Adeoya-Osiguwa et al., 2003), further supporting the importance of these steroids in the acquisition of sperm fertilizing capability (Ded et al., 2010; Guido et al., 2011; Sebkova et al., 2012). Indeed, the ejaculate contains uncapacitated spermatozoa which, being in a quiescent state, do not possess the ability to fertilize an oocyte; they acquire this ability in the female reproductive tract, through a process known as capacitation (Travis and Kopf, 2002; Suarez, 2008), which enables the sperm to bind to the zona pellucida of the oocyte and to undergo the acrosome reaction (Suarez, 2008). The biochemical changes induced by estrogens during capacitation occur rapidly, addressing the nongenomic action of ERs, which might represent an exclusive modality of ER action in spermatozoa since sperm are considered transcriptionally inactive (Aquila et al., 2004). Through its ER, the estrogen 17-β-estradiol (E2) stimulates other important sperm functions, including motility and longevity, by activating the phosphatidylinositol-3-OH kinase (PI3K)/AKT pathway (Adeoya-Osiguwa et al., 2003; Aquila et al., 2003).

Besides estrogens, many nonsteroidal compounds in the environment have been found to possess estrogenic activity. Phytoestrogens such as genistein (found in soy and legumes), quercetin (found in parsley and red wine), and 8-prenylnaringenin (found in hops and beer) are widespread in the diet. Environmental estrogens have the potential to alter reproductive hormones, spermatogenesis, sperm capacitation, and fertility (Adeoya-Osiguwa et al., 2003; Fraser et al., 2006), and may act as ''endocrine disruptors'' when used at relatively high concentrations (Skakkebaek et al., 2001). To date, few studies designed to evaluate the effects of phytoestrogens on fertility or reproductive hormones in human males are available (Cederroth et al., 2010). Several phytoestrogens have been tested, but the effects of Myricetin (3,3′,4′,5′,5,7-hexahydroxy-2-phenylchromen-4-one) on reproductive parameters has never been addressed. Myricetin is a naturally occurring flavonol, structurally similar to quercetin, which is found in significant amounts in berries, tea, and red wine (Huang et al., 2010). Controversial data are available in the literature on the therapeutic potential of Myricetin since it has been reported to possess both antioxidant (Sellappan and Akoh, 2002; Lu et al., 2006) and pro-oxidant activity in vitro (Chobot and Hadacek, 2011), as well as both anti-carcinogenic (He et al., 2008) and pro-tumorigenic properties (Maggiolini et al., 2005). Since it has been shown that Myricetin elicits estrogenic activity (Maggiolini et al., 2005) and that estrogens are able to stimulate sperm capacitation and the consequent biochemical changes (e.g., increased metabolic rate and overall energy expenditure (Visconti et al., 1998; Baldi et al., 2000; Aquila et al., 2005b)), it is important to determine if this flavonol affects the mature sperm function in a manner that can alter fertility. Thus, we assessed the influence of different concentrations of Myricetin on several aspects of sperm biology. In addition, as there is a close link between energy balance and reproduction (Chehab, 2000) and sperm cells are able to modulate their own metabolism independently of systemic regulation (Aquila et al., 2005b), the action of Myricetin on lipid and glucose metabolism in human sperm was also evaluated.

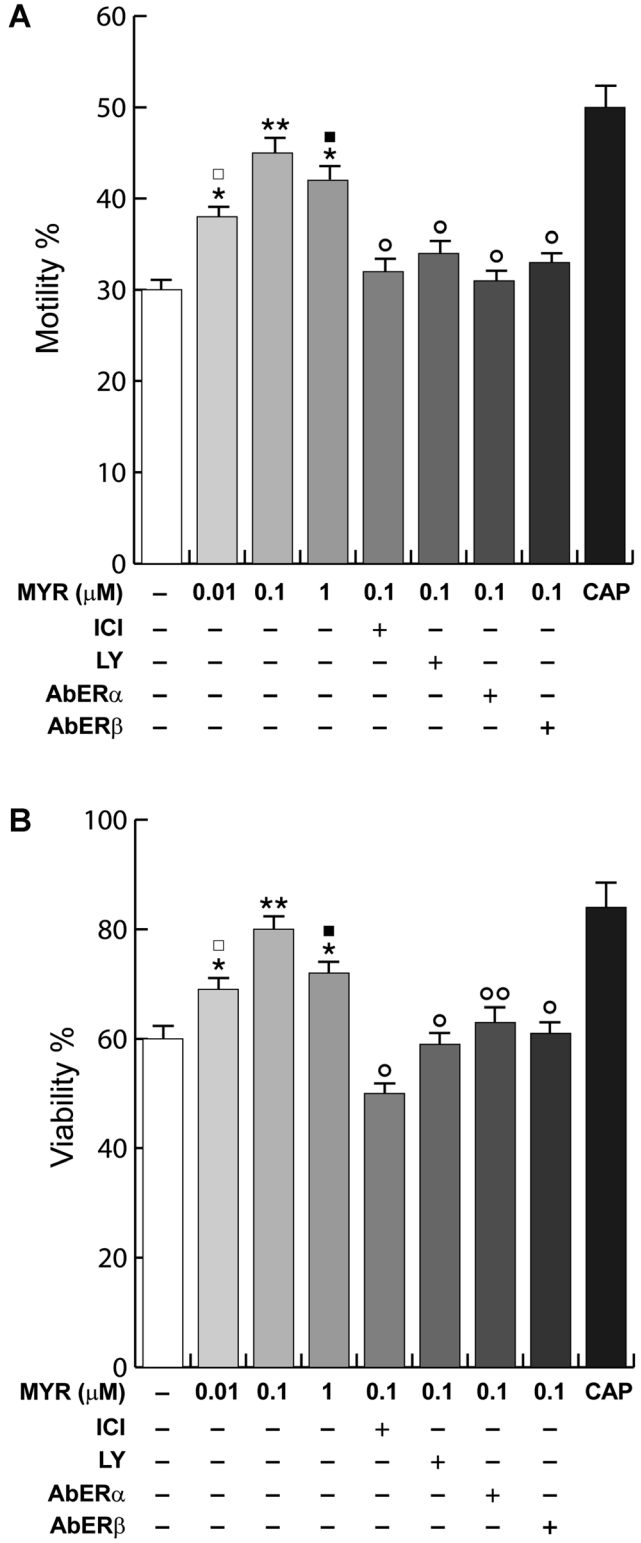
RESULTS

Myricetin Increases Motility and Viability of Human Sperm

To assess the effect of Myricetin on sperm motility, we subjected sperm samples from human normozoospermic donors to increasing concentrations of the phytoestrogen (10 nM, 100 nM, 1 μM). Following stimulation with 10 and 100 nM Myricetin, we observed a 25% and 50% increase in sperm motility, respectively, compared to that of an uncapacitated control samples. In particular, the effect at 100 nM concentration was similar to that of a capacitated sample used as positive control (bovine serum albumin (BSA), see the Materials and Methods Section), while a higher amount (1 μM) instead resulted in a slight decrease of motility respect to the 100 nM Myricetin-treated sample (Fig. 1A). A similar trend was observed in sperm viability experiments, with an increase of ~20% over uncapacitated control in 10 nM and 1 μM Myricetin-treated samples, and of ~30% in sperm stimulated with 100 nM Myricetin, a value comparable to that obtained in the capacitated control sample (Fig. 1B).

We hypothesized that both the ''classical'' ERs could mediate Myricetin estrogenic actions in sperm. Therefore, cells were first pre-treated with the pure anti-estrogen compound ICI 182.780 (ICI), which can bind to both ER subtypes (Paige et al., 1999), and then treated with 100 nM Myricetin. This co-treatment abrogated the effects of 100 nM Myricetin on both sperm motility and viability (Fig. 1A,B). To better define the role of ER isoforms in mediating Myricetin responses, we added selective

estrogen receptor antibodies, directed against either ERα (AbERα) or ERβ (AbERβ), to the 100 nM Myricetin-treated samples. Both antibodies reversed the effects of the flavonol on sperm motility and viability. Finally, to assess if the PI3K pathway is involved in Myricetin action, we co-treated sperm cells with the PI3K inhibitor LY294002 (LY). Indeed, LY completely abolished the positive effects induced by 100 nM Myricetin (Fig. 1A,B).



Effects of Myricetin on Cholesterol Efflux and Protein Tyrosine Phosphorylation in Human Spermatozoa

We next investigated the influence of Myricetin on membrane cholesterol efflux and on the tyrosine phosphorylation status of specific sperm proteins involved in the capacitation process (Visconti et al., 2002). As reported in Figure 2A, all tested doses of Myricetin induced a significant incremental increase in cholesterol efflux, reaching almost a two-fold increase in the 100 nM Myricetin-treated versus untreated sample. This value was comparable to that observed in a capacitated sample used as positive control; lower (10 nM) and higher (1 μM) concentrations of Myricetin were slightly less effective than 100 nM. Co-treatment with ICI, AbERs, or LY abrogated the effect of 100 nM Myricetin (Fig. 2A). Moreover, the phytoestrogen dramatically induced protein tyrosine phosphorylation, as shown by the very strong bands observed at 95–97 kDa, which are reported to be elevated during capacitation (Naz, 1996). One hundred nanomolar Myricetin elicited the maximum effect (threefold increase over control), which was abrogated by ICI, AbERs, or LY (Fig. 2B). Interestingly, this same concentration of phytoestrogen was almost as effective as capacitating conditions on eliciting tyrosine phosphorylation, while lower and higher doses showed a weaker effect (Fig. 2B).

Myricetin Triggers the Acrosome Reaction in Ejaculated Sperm

We asked if Myricetin could affect acrosin activity, a metric of the sperm fertilizing capacity (Tummon et al., 1991), in human ejaculated sperm. Indeed, in samples treated with low concentrations (10 and 100 nM) of the phytoestrogen, we observed a significant dose-dependent increase of acrosin enzymatic activity compared to control samples. Following treatment with 100 nM Myricetin, we

Figure 1. Myricetin increases sperm motility and viability. Spermatozoa were incubated in un-supplemented Earle's medium (uncapacitating medium) for 30 min at 37°C and 5% CO_2, in the absence (–) or presence of increasing concentrations of Myricetin (0.01–1 μM). Additionally, the anti-estrogen ICI 182,780 (ICI) (1 μM), the PI3K inhibitor LY294002 (LY) (10 μM), or antibodies against ERα and ERβ (AbERα or AbERβ, respectively; both 1:100 dilution) were added to 0.1 μM Myricetin treated samples. Spermatozoa incubated in capacitating medium (BSA) were used as positive control (CAP). Sperm motility (A) and viability (B) were assessed microscopically, as described in the Materials and Methods Section, and the values are expressed as percentage of motile sperm or viable cells, respectively. Columns represent mean ± standard deviation of four independent experiments, each done in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated (–); $^{\circ\circ}P < 0.05$ and $^{\circ}P < 0.01$ versus 0.1 μM Myricetin; $^{\square}P < 0.01$ and $^{\blacksquare}P < 0.05$ versus CAP.

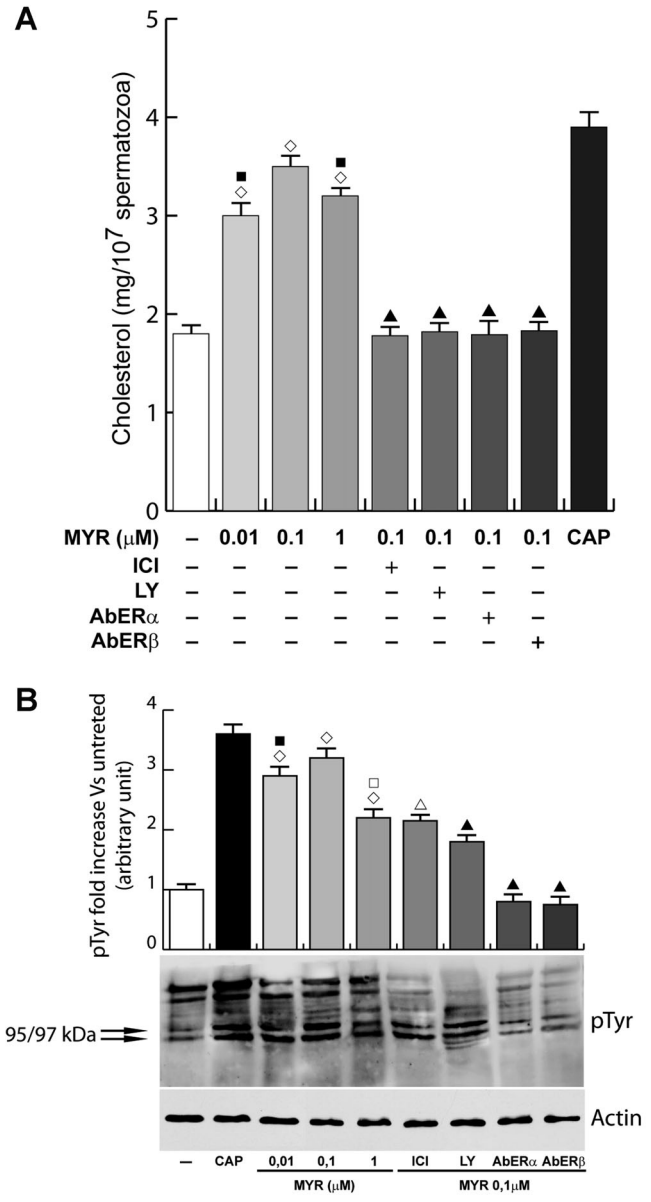


Figure 2. Effects of Myricetin on cholesterol efflux and protein tyrosine phosphorylation in human spermatozoa. Spermatozoa were incubated in un-supplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (–) or presence of increasing concentrations of Myricetin (0.01–1 μM), or 0.1 μM Myricetin plus the anti-estrogen ICI 182,780 (ICI) (1 μM), the PI3K inhibitor LY294002 (LY) (10 μM), or antibodies against ERα and ERβ (AbERα and AbERβ, respectively; both 1:100 dilution). Spermatozoa incubated in capacitating medium (BSA) were used as positive control (CAP). **A**: Cholesterol in culture medium was measured by enzymatic colorimetric assay (detailed in the Materials and Methods Section). Columns report mean ± standard deviation of six independent experiments performed in duplicate. **B**: Total proteins (80 μg) were used for Western blot analysis to assess tyrosine phosphorylation (pTyr). Actin was used as loading control. Densitometric analysis (mean ± standard deviation) of the 95-kDa pTyr band/actin is reported. The results are representative of four independent experiments. $^{\diamond}P < 0.001$ versus untreated (–); $^{\triangle}P < 0.01$ and $^{\blacktriangle}P < 0.001$ versus 0.1 μM Myricetin; $^{\square}P < 0.01$ and $^{\blacksquare}P < 0.05$ versus CAP.

observed a 70% increase over untreated samples, which almost mimicked the effect of the capacitated sample used as positive control. No further increase was observed at the highest concentration (1 μM) tested, while co-treatments with ICI, AbERs, or LY counteracted the effect induced by 100 nM Myricetin (Fig. 3).

Myricetin Activates the PI3K Pathway in Human Spermatozoa

Since E2 induces phosphorylation of the proteins involved in the PI3K/AKT pathway (Aquila et al., 2004), we examined the effects of Myricetin on tyrosine phosphorylation of the pro-survival protein AKT. We found the highest levels of phosphorylated AKT (pAKT) in 100 nM

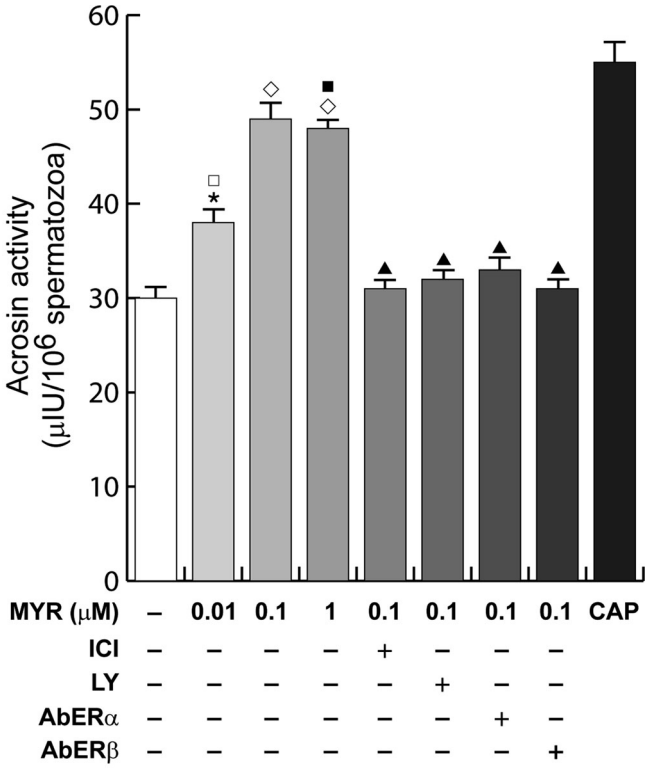


Figure 3. Myricetin triggers the acrosome reaction in ejaculated sperm. Washed spermatozoa were incubated under non-capacitating conditions for 30 min at 37°C and 5% CO_2 in the absence (–) or presence of either increasing concentrations of Myricetin (0.01–1 μM), or 0.1 μM Myricetin plus the anti-estrogen ICI 182,780 (ICI) (1 μM), the PI3K inhibitor LY294002 (LY) (10 μM), or antibodies against ERα and ERβ (AbERα and AbERβ, respectively; both 1:100 dilution). Spermatozoa incubated in capacitating medium (BSA) were used as positive control (CAP). Acrosome reaction was determined as described in the Materials and Methods Section, and the results are expressed as values of acrosin activity. Columns represent mean ± standard deviation of four independent experiments, each done in duplicate. $^{*}P < 0.05$ and $^{\diamond}P < 0.001$ versus untreated (–); $^{\blacktriangle}P < 0.001$ versus 0.1 μM Myricetin; $^{\square}P < 0.01$ and $^{\blacksquare}P < 0.05$ versus CAP.

Myricetin-treated samples (almost fivefold over the untreated control). This increase occurred without altering protein content, as evidenced by the steady level of the housekeeping protein actin, and was comparable to that observed in the capacitated sample used as a positive control. In line with the other results presented here, lower (10 nM) and higher (1 μM) concentrations of flavonol were not as effective (only about threefold increase over untreated), and the combination with ICI, AbERs, or LY completely abrogated the phosphorylation induced by 100 nM Myricetin (Fig. 4).

Myricetin Influences Both Glucose and Lipid Metabolism in Human Sperm

PI3K/AKT signaling is an important node in cellular metabolism as well as in survival. We therefore tested the effect of Myricetin on both glucose and lipid metabolism in human sperm. Treatment of ejaculated spermatozoa with

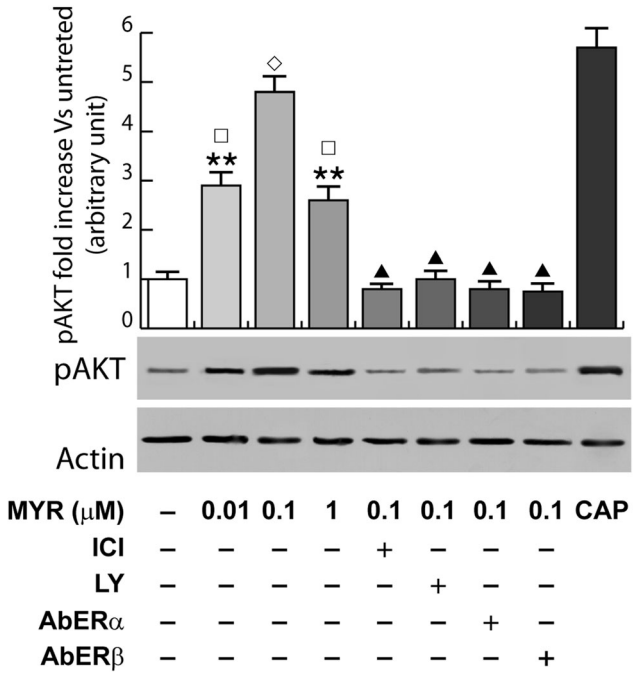


Figure 4. Myricetin activates the PI3K pathway in human spermatozoa. Washed spermatozoa were incubated under non-capacitating conditions for 30 min at 37°C and 5% CO_2 in the absence (−) or presence of either increasing concentrations of Myricetin (0.01 –1 μM), or 0.1 μM Myricetin plus the anti-estrogen ICI 182,780 (ICI) (1 μM), the PI3K inhibitor LY294002 (LY) (10 μM), or antibodies against ERα and ERβ (AbERα and AbERβ, respectively; both 1:100 dilution). Spermatozoa incubated in capacitating medium (BSA) were used as positive control (CAP). Protein lysates (80 μg) were subjected to Western blot for detection of the indicated proteins. Actin was used as a loading control. Densitometric analysis (mean ± standard deviation) of three independent experiments is reported as pAkt/Actin relative intensity. $^{**}P < 0.01$ and $^{\diamond}P < 0.001$ versus untreated (−); $^{\blacktriangle}P < 0.001$ versus 0.1 μM Myricetin; $^{\square}P < 0.01$ versus CAP.

10 nM Myricetin induced only a slight increase in glucose-6-phosphate dehydrogenase (G6PDH) activity, while higher concentrations (100 nM to 1 μM) were strikingly more effective (over threefold induction compared to uncapacitated control). In particular, the 100 nM dose led to the highest enzymatic activity, which was similar to that detected in a capacitated sample used as positive control, although not significantly higher than that induced by 1 μM Myricetin. Moreover, ICI and both AbERs reversed the 100 nM Myricetin-mediated enzymatic induction; LY, however, was not able to counteract the flavonol-effect (Fig. 5A).

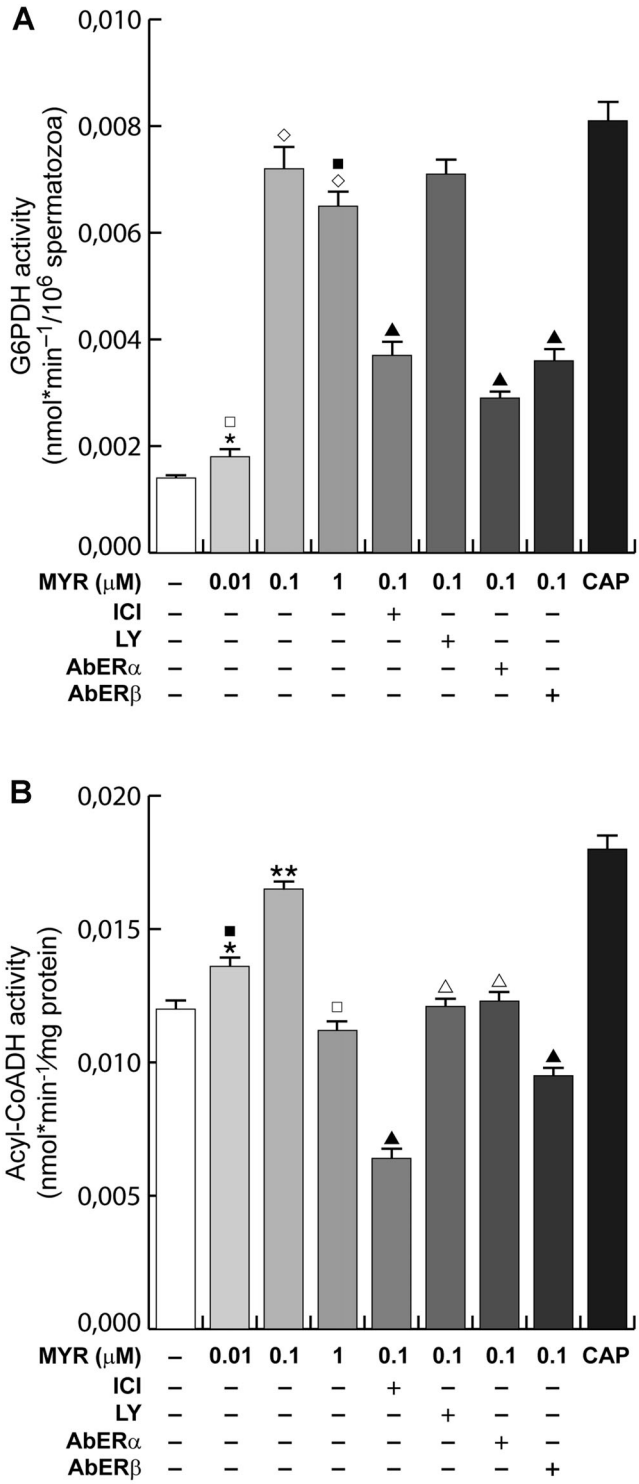
We tested fatty acid β-oxidation by measuring acyl-CoA dehydrogenase activity in ejaculated sperm subjected to Myricetin stimulation to gain more insight into sperm energy management. Our results show that Myricetin exerts significant effects on the enzymatic activity at low concentrations (10–40% increase over control in 10 nM- and 100 nM-treated samples, respectively), whereas the highest concentration (1 μM) did not produce any relevant change compared to the untreated sample. Once again, 100 nM Myricetin was the most effective dose tested since the induction of the acyl-CoA dehydrogenase activity was comparable to that observed in a capacitated sample. ICI, AbERs, and LY co-treatment counteracted this induction, with ICI and AbERβ showing the highest inhibition (Fig. 5B).

DISCUSSION

Phytoestrogens are plant compounds structurally similar to E2 that can interact with estrogen receptors (ERs) to promote and/or inhibit estrogenic responses (De Amicis et al., 2011a). In recent years, the biological properties of these natural estrogen-like compounds have attracted increasing interest for the potential beneficial effects on human health (Patisaul and Jefferson, 2010; Pilsakova et al., 2010). In this regard, a reduced incidence and mortality from a variety of diseases has been associated with moderate consumption of red wine (Cornwell et al., 2004), which is considered a major source of estrogenic compounds, including the more-abundant Myricetin (Waterhouse, 2002). Yet the effects of Myricetin on male gamete physiology have never been addressed. It is well documented that E2 and environmental estrogens mediate mammalian sperm capacitation, the acrosome reaction, and fertilizing ability through ERs (Adeoya-Osiguwa et al., 2003; Jefferson et al., 2012). As Myricetin has been reported to act as an agonist for ERs in human breast cancer cells (Maggiolini et al., 2005), we asked if this phytoestrogen could influence sperm biology and whether or not the mechanism occurs through the ERs.

First, we tested the effects of Myricetin on two important features of sperm biology: motility, the ability of the male gametes to properly "swim" towards an oocyte, and viability, an important evolutionary adaptation in humans that determines fertilization success by conferring the potential to "wait" until the egg is released (Aquila et al., 2008). From our data it emerged that an acute (30 min) exposure to low concentrations of Myricetin is able to improve sperm motility

and survival, whereas higher concentrations tend to be less effective. Longer or even chronic exposure to Myricetin on sperm motility and survival were not tested, however, and need to be addressed in future studies.



Capacitation is a functional maturation of the spermatozoon that allows the sperm to achieve competency to fertilize an egg. It occurs in the female reproductive tract through a series of physiological changes, such as increased cholesterol efflux, tyrosine phosphorylation, as well as increased rate in glucose and lipid metabolism (Aquila et al., 2006, 2009, 2010; Guido et al., 2011; De Amicis et al., 2011b). Here we demonstrated that the estrogen mimetic Myricetin increases cholesterol efflux, an event that initiates capacitation and, in turn, induces the phosphorylation of a set of specific proteins in the molecular range of 40–120 kDa sperm proteins (Visconti et al., 2002). This result fits well with our previous observations showing that ER activation induces tyrosine phosphorylation in human sperm (Aquila et al., 2004).

Capacitation prepares sperm that are in close proximity of the oocyte to be responsive to the physiologic stimulus that triggers the acrosome reaction. This consists of the release of different enzymes, mainly acrosin, which facilitates the penetration of sperm through the zona pellucida to reach the egg. In our experimental conditions Myricetin significantly increased the activity of acrosin.

It is worth noting that the strongest activity on sperm capacitation-associated features (motility, vitality, cholesterol efflux, protein phosphorylation, and acrosin activity) was obtained with 100 nM Myricetin treatment, while the use of higher doses (1 μM) did not exert any further improvement, but rather tended to diminish the effects. Notably, in all experiments, the effect of 100 nM Myricetin was comparable to that of the capacitating agent BSA, used as positive control, suggesting that an optimal dose of the flavonol might trigger the above-mentioned capacitation-associated physiological changes, while, in contrast, exposure to higher concentrations of the phytoestrogen could lead to a reduced response to the stimulus. Since Myricetin has been reported to act as an agonist for ERs in human cancer cells (Maggiolini et al., 2005), these results could be explained by the assumption that Myricetin exerts its effects in human sperm through a mechanism involving ER signaling, and that the lower activity observed at higher

Figure 5. Myricetin influences both glucose and lipid metabolism in human sperm. Sperm samples, washed twice with an non-capacitating medium, were incubated in the same medium for 30 min at 37°C and 5% CO_2, and treated in the absence (–) or presence of increasing concentrations of Myricetin (0.01–1 μM), or 0.1 μM Myricetin plus the anti-estrogen ICI 182,780 (ICI) (1 μM), the PI3K inhibitor LY294002 (LY) (10 μM), or antibodies against ERα and ERβ (AbERα and AbERβ, respectively; both 1:100 dilution). Spermatozoa incubated in capacitating medium (BSA) were used as positive control (CAP). **A**: The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm every 20 sec for 1.5 min. Data are expressed in nmol $min^{-1}/10^6$ spermatozoa. **B**: Sperm protein lysates were subjected to the assay of Acyl-CoA dehydrogenase (Acyl-CoADH) (see the Materials and Methods Section) and expressed as nmol min^{-1}/mg protein. Columns are mean ± standard deviation and the data reported are representative of six independent experiments performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$, and $^{\diamond}P < 0.001$ versus untreated (–); $^{\triangle}P < 0.01$ and $^{\blacktriangle}P < 0.001$ versus 0.1 μM Myricetin; $^{\square}P < 0.01$ and $^{\blacksquare}P < 0.05$ versus CAP.

concentrations could be due to an over-stimulation of the receptor. In line with this assumption, it was recently reported that the capacitating ability of sperm can be significantly lowered by increased levels of estrogens. In fact, although estrogens exert a time- and concentration-dependent stimulatory effect on sperm tyrosine phosphorylation during capacitation, the corresponding number of sperm that undergo the acrosome reaction is lower (Ded et al., 2010; Sebkova et al., 2012). The observation that concomitant treatment with the anti-estrogen ICI or with antibodies directed against either ERα or ERβ dramatically reduce Myricetin activity, confirmed the involvement of both ERs in the flavonol-mediated effects. ER signaling is not the only pathway triggered by Myricetin, however. Indeed, PI3K signaling is also involved since the PI3K inhibitor LY was able to abrogate Myricetin-induced events, including the activation of the PI3K downstream target AKT, whose phosphorylation status returned to basal levels following the inhibition of both PI3K and ERs pathways. These results confirm that the mechanism through which Myricetin acts on sperm survival occurs via the ERs/PI3K axis, and are in line with our previous findings showing that E2 exposure enhances phosphorylation of proteins belonging to the PI3K/AKT pathway and that these phosphorylations are reduced by ICI and LY treatment (Aquila et al., 2004). The pro-survival effect of Myricetin, acting through AKT activation, was recently reported in Chinese hamster lung fibroblast and in skeletal muscles of fructose-fed rats (Kang et al., 2010; Tzeng et al., 2011), although this might not represent the general mechanism since other studies reported a pro-apoptotic effect of comparable doses of Myricetin, achieved through AKT inhibition (Kumamoto et al., 2009; Kim et al., 2010).

Capacitated sperm display an increased metabolism and overall energy expenditure, presumably to sustain all the aforementioned physiological changes. The effect of glucose on the fertilizing ability of sperm appears to be mediated by the production of NADPH during the enzymatic reaction of glucose-6-phosphate, catalyzed by G6PDH, the rate-limiting enzyme in the pentose phosphate pathway (PPP) (Aquila et al., 2009). NADPH, in turn, is essential for fatty acid synthesis from acetyl-CoA. Fatty acids have two possible fates: β-oxidation (which greatly increases during capacitation) to produce ATP or re-esterification, back into triacylglycerol (Ando and Aquila, 2005).

Myricetin has been reported to exert an insulin-mimetic effect on lipogenesis and glucose transport in adipocytes of rats with noninsulin-dependent diabetes mellitus (Ong and Khoo, 1996) to improve glucose utilization, thereby lowering the plasma glucose levels in type 1 diabetes-like animal models (Liu et al., 2005), and to ameliorate insulin sensitivity in animals exhibiting insulin resistance (Liu et al., 2007) as well as in fructose-fed rats with high-glucose plasma levels (Tzeng et al., 2011), although recent studies also reported inhibitory effects on G6PDH activity (De Abreu et al., 2011). Myricetin seems to play an important role in lipid metabolism as well, since it was able to suppress body-fat accumulation by increasing fatty acid β-oxidation, which was likely mediated via up-regulation of PPARα expression in the liver of high fat diet-fed rats (Chang et al., 2012). In line with these observations, our results show how low doses of Myricetin are able to induce both glucose and lipid metabolism, and thus energy expenditure, by stimulating G6PDH activity and fatty acid β-oxidation, respectively. These data are consistent with our previous reports demonstrating that, in ejaculated sperm, E2/ERs are able to induce both enzymatic activities (Guido et al., 2011). Indeed, the effects of Myricetin on sperm metabolism occur through the activation of the ER pathway, while the PI3K/AKT signaling, which is well established to be involved both in glucose and lipid metabolism (Saltiel and Kahn, 2001), seems to be important only in Myricetin-induced fatty acid β-oxidation, since LY was not able to counteract the flavonol mediated increase in G6PDH activity. It is worth noting that, once again, 100 nM Myricetin displays the strongest effect on both glucose and lipid metabolism, acting as a capacitating agent. At higher concentrations, this efficacy is reduced or completely lost, corroborating the idea that, in target cells, polyphenols show opposite behaviors, eliciting oxidant/toxic actions (Chen et al., 2003) at higher doses, while exerting an antioxidant/protective role at lower doses (Spinaci et al., 2008; De Amicis et al., 2012). As concomitant exposure to both E2 and environmental estrogens has been reported to promote ''overcapacitation'' and the acrosome reaction, and prematurely acrosome-reacted sperm are nonfertilizing (Yanagimachi, 1994), similar untimely responses occurring in vivo could reduce the number of potentially fertilizing cells and thus have a detrimental effect on fertility.

Considering Myricetin and quercetin represent the 20–50% of the total flavonol content in red wines (Waterhouse, 2002), which ranges from 53 to 200 mg/L (Ritchey and Waterhouse, 1999), even daily consumption of moderate amounts of red wine (1–2 glasses) may be sufficient to increase serum levels of Myricetin into the micromolar range investigated in our study. As capacitation and fertilization occur in the female reproductive tract, where sperm are exposed to high estrogen levels, the co-estrogenic action of circulating Myricetin could be harmful to sperm function.

In conclusion, we have provided the first mechanistic insight into the estrogenic potential of Myricetin on motility, survival, capacitation, acrosome reaction, and metabolism of human sperm. Low concentrations of Myricetin might improve motility, viability, and energy expenditure in the human male gamete, while higher amounts seem to reduce the efficacy exerted by lower doses. Further investigation is needed to better define the effects that high doses of Myricetin, which could derive even from moderate red wine consumption, may exert on human male gamete function and, therefore, on fertility.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA) protein standard, Laemmli sample buffer, prestained molecular weight markers,

Percoll (colloidal PVP coated silica for cell separation), phosphate-buffered saline (PBS), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide (DMSO), Earle's balanced salt solution, 3,3′,4′,5′,5,7-hexahydroxy-2-phenylchromen-4-one, Myricetin, octanoyl-CoA, Mops, FAD+, LY294002 (LY), and all other chemicals were purchased from Sigma Chemical (Milan, Italy). ICI 182,780 (ICI) was purchased from Zeneca Pharmaceuticals (Cheshire, UK). Monoclonal mouse antibody (Ab) to human ERalpha (F-10) (AbERα); rabbit polyclonal Ab to human ERbeta (H-150) (AbERβ); rabbit anti-p-AKT1/AKT2/AKT3; rabbit anti-phosphotyrosine Ab (PY99); goat polyclonal actin Ab (1–19); peroxidase-coupled anti-mouse, anti-rabbit, and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). Enhanced chemiluminescence (ECL) Plus Western blotting detection system, Hybond™ ECL™, and Hepes Sodium Salt were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Cholesterol–oxidase–peroxidase (CHOD-POD) enzymatic colorimetric assay kit and glucose-6-phosphate dehydrogenase (G6PDH) activity assay kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Myricetin and ICI were dissolved in ethanol (0.02% final concentration in culture); solvent controls did not induce any positive result in all in vitro assays (data not shown).

Semen Samples and Spermatozoa Preparations

Semen specimens were obtained from normozoospermic donors, after 3 days of sexual abstinence. The samples were collected in sterile containers, and left for 30 min to completely liquefy before being processed. Sperm samples with normal parameters of semen volume, sperm count, motility, vitality, and morphology, according to the World Health Organization Laboratory Manual (Cardona Maya, 2010), were pooled and included in this study. Spermatozoa preparations were performed as previously described (Aquila et al., 2010). The study was approved by the local medical–ethical committee of the University of Calabria, and all participants submitted informed consent.

Processing of Ejaculated Sperm

For each experiment, three normozoospermic samples were used. After liquefaction, semen samples were pooled and then subjected to centrifugation (800*g*) on a discontinuous Percoll density gradient (80:40%, v/v) (Aquila et al., 2002). The 80% Percoll fraction was examined using an optical microscope equipped with a 100× oil objective to ensure that a pure sample containing only spermatozoa was obtained. Samples of Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium), and were incubated in unsupplemented Earle's balanced salt solution for 30 min at 37°C and 5% CO_2, with or without the following treatments: increasing Myricetin concentrations (10 nM, 100 nM, and 1 μM) or 100 nM Myricetin combined with ICI (1 μM), AbERα, AbERβ (both 1:100 dilution in each experiment), or LY (10 μM). When the cells were treated with ICI or LY, a pretreatment of 30 min was performed. As a positive control, samples were incubated in Earle's balanced salt solution medium supplemented with 600 mg BSA and 200 mg sodium bicarbonate per100 ml (capacitating medium).

Western Blot Analysis of Sperm Proteins

Sperm samples, washed twice with non-capacitating medium, were incubated in the absence or presence of the indicated treatments, and then centrifuged for 5 min at 5,000*g*. The pellet was resuspended in lysis buffer and processed as previously described (De Amicis et al., 2012). We used 80 μg of proteins for Western blot analysis. As an internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first Ab and reprobed with anti-β actin. The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700, Bio–Rad, Hercules, CA).

Evaluation of Sperm Motility and Viability

Sperm motility was assessed by means of light microscopy with a Mackler Counting Chamber, as previously described (Aquila et al., 2010), and was expressed as percentage of motile sperm.

Viability was assessed by the red eosin exclusion test, using Eosin Y (Aquila et al., 2005a). Spermatozoa were washed in uncapacitating medium and centrifuged at 800*g* for 20 min. Ten microliters of Eosin Y (0.5% in PBS) were mixed with an equal volume of sperm sample on a microscope slide. An independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells), and sperm viability was expressed as percentage of alive sperm.

Measurement of Cholesterol in the Sperm Culture Medium

Percoll-purified sperm samples, were incubated in uncapacitating medium for 30 min at 37°C and 5% CO_2 in the absence or presence of increasing Myricetin concentrations (10 nM to 1 μM). Other samples were incubated 100 nM Myricetin combined with ICI (1 μM), AbERα, AbERβ (both 1:100 dilution), or LY (10 μM). At the end of the incubation, cholesterol was measured in duplicate by a CHOD-POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium, as previously described (Aquila et al., 2006; De Amicis et al., 2011b; Guido et al., 2011).

Acrosin Activity Assay

Acrosin activity was assessed by the method of Kennedy et al. (1989) and as previously described (Aquila et al., 2003, 2006). Briefly, Percoll-purified sperm were washed in Earle's balanced salt solution medium supplemented with $CaCl_2$ (266 mg/100 ml), BSA (600 mg/100 ml), sodium

pyruvate (3 mg/100 ml), sodium lactate (360 ml/100 ml), and sodium bicarbonate (200 mg/100 ml), then centrifuged at 800*g* for 20 min. Sperm were resuspended and treated as indicated. Then 1 ml of substrate ± detergent mixture (23 mmol/L BAPNA in DMSO and 0.01% Triton X-100 in 0.055 mol/L NaCl, 0.055 mol/L HEPES at pH 8.0, respectively) for 3 hr at room temperature was added. Aliquots (50 ml) were removed at 0 and 3 hr, and the percentages of viable cells were determined. After incubation, 0.5 mol/L benzamidine was added (0.1 ml) to each of the tubes and then centrifuged at 1,000*g* for 30 min. The supernatants were collected and the acrosin activity measured spectrophotometrically at 410 nm.

Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity Assay

Spermatozoa samples were incubated in non-capacitating medium for 30 min at 37°C and 5% CO_2 in the presence or absence of the indicated treatments. After incubation, 50 μL of sperm extracts were diluted in opportune buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/ml glucose-6-phosphate, 10 mg/ml $NADP^+$, pH 7.6) for spectrophotometric determination. The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm, as previously described (Aquila et al., 2009), and reported as nmol $min^{-1}/10^6$ spermatozoa.

Acyl-CoA Dehydrogenase Activity Assay

An assay of acyl-CoA dehydrogenase (Acyl-CoADH) was performed on sperm incubated in the indicated treatments, using a modification of the method described by Lehman et al. (1990) as previously reported (Aquila et al., 2010). In brief, after lysis, 70 μg of sperm protein was added to buffer containing 20 mM Mops, 0.5 mM EDTA, and 100 mM FAD^+ at pH 7.2. Reduction of FAD^+ to FADH was read at 340 nm upon addition of octanoyl-CoA (100 mM) every 20 sec for 1.5 min. Data are expressed in nmol min^{-1}/mg protein. The enzymatic activity was determined with three control media: one without octanoyl-CoA as substrate, one without the co-enzyme (FAD^+), and the third without either substrate or co-enzyme (data not shown). Every experiment was performed six times, in duplicate within each experiment.

Statistical Analysis

The data obtained were presented as the mean ± standard deviation of at least three independent experiments. The differences in mean values were calculated using analysis of variance (ANOVA), with a significance level up to $P \leq 0.001$.

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ORIGINAL ARTICLE

Human Sperm Anatomy: Different Expression and Localization of Phosphatidylinositol 3-Kinase in Normal and Varicocele Human Spermatozoa

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ABSTRACT

Recent reports support the possible role of PI3K in sperm capacitation and acrosome reaction, although studies regarding PI3K identity in human sperm, under certain disease states such as varicocele, are still lacking. The authors, therefore, examined the expression profile and ultrastructural localization of PI3K in human semen samples, comparing healthy donors and patients with varicocele. The results obtained performing western blotting assay showed decreased PI3K expression in varicocele with respect to the "healthy" sperm. Immunogold labeling revealed human sperm cellular compartments containing PI3K, evidencing it in the head at both the membrane and nucleus and the entire tail, from the middle to the end piece of normal sperm. In varicocele PI3K label was confined to the head, with a strong reduction of specific reaction in the neck, middle piece, and tail. In conclusion, the data suggest that PI3K may play a role in the maintenance of male factor infertility associated with varicocele, and it may be further exploited as an additional molecular marker for the diagnosis of male infertility disorders.

Keywords: Human spermatozoa, infertility, male reproduction

Varicocele of spermatic veins is considered to be one of the major causes of male infertility. The incidence of varicocele in the adult normal male population is approximately 15%, and it has has been implicated as a cause of primary infertility in 35–50% of patients and up to 81% of men with secondary infertility. The surprisingly high incidence in secondary infertility suggests that varicoceles cause progressive decline in testicular function over time [1]. In fact, the association of varicocele with decreased testicular size and abnormal testicular histology is clearly established [2]. Despite many studies concerning varicocele in male fertility, the exact mechanism of the varicocele's effect on sperm function remains to be defined. In addition, the molecular alterations influencing fertilization process in varicocele are not clearly established.

The ability to fertilize an oocyte of human spermatozoa is acquired in the female reproductive tract. This process is known as capacitation. Capacitation enables the sperm to undergo the acrosome reaction to aid the oocyte–sperm fusion process [3,4].

Motility is a peculiar function of the mature male gamete. In mammalian spermatozoa, the ability to actively swim, based on the specialized structure of the flagellum, is acquired during the transit through the epididymis under the control of different factors [5–8].

It has recently been suggested that phosphatidylinositol 3-kinase (PI3K) plays an important role in regulation of sperm capacitation and motility [9]. PI3K is an important intracellular mediator of cell survival and antiapoptotic signals. It belongs to a family of dimeric enzymes, consisting of catalytic (110 kDa) and regulatory subunits (85 kDa), and it is widely expressed in somatic cells.

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It was reported that PI3K is involved in actin polymerization [10], a process that must occur in sperm capacitation [11] and in the regulation of sperm motility [12–15]. We previously evidenced PI3K expression in human sperm [16–19] and we also demonstrated that estrogens via ERalpha and ERbeta activate the PI3K/Akt pathway controlling human sperm survival and metabolism. Similarly, in uncapacitated sperm, both insulin and leptin increased PI3K activity [17,18], thereby possibly modulating the availability of the spermatozoa's energetic substrates during capacitation, since the PI3K pathway is involved in the autonomous regulation of glucose metabolism in sperm. Besides, our findings demonstrated that PI3K mediates androgens/AR effects and interferes with sperm survival [20]. Recently the class I PI3K has been shown to be an important mediator of acrosome reaction induced by ZP3 in mouse sperm [21].

Given the demonstrated involvement of the PI3K in sperm physiology, in the present study we aimed to investigate the specific cellular compartment where the enzyme is precisely located. To shed light on the pathophysiology of varicocele, by identifying factors involved in the effects of this disease on sperm fertilization potential, we compared the results obtained from healthy sperm with varicocele sperm.

MATERIALS AND METHODS

Chemicals

Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide, Earle's balanced salt solution, and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus western blotting detection system, Hybond TM ECL TM, and HEPES sodium salt were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Rabbit polyclonal antibody raised against a recombinant protein corresponding to aa 189–390 of human PI3K (p110 subunit) and peroxidase-coupled anti-rabbit IgG secondary Ab were from Santa Cruz Biotechnology (Heidelberg, Germany). Colloidal gold conjugated anti-rabbit IgG secondary Ab was from Sigma Chemical.

Semen Samples and Spermatozoa Preparations

Human semen was collected, according to the World Health Organization (WHO)-recommended procedure [22], by masturbation from healthy volunteer donors of proven fertility. Spermatozoa preparations were performed as previously described [23,24]. Briefly, semen samples with normal parameters of volume, sperm count, motility, morphology, and vitality, according to the *WHO Laboratory Manual* [22], were included in this study. Varicocele samples of patients who consulted us for fertility investigation were also placed in our study. Reflux of blood in the pampiniform plexus was determined by palpation employing the Valsalva maneuver. Physical examination is the reference standard to diagnose varicoceles in subfertile men. Varicocele samples used in this study were from patients with diagnosed varicocele of grade III (visible without palpation) on the left testis, and their ejaculates were found to have total sperm count of 13×10^6 sperm cells per ejaculate, percentage of motility PR + NP of 38%, percentage of normally formed features of 20%, and viability percentage of 60%. Samples of oligoasthenoteratozoospermic (OAT) patients without varicocele, but with similar semen characteristics with respect to those with varicocele, were used as control samples in our study to isolate a specific effect of varicocele. The study was approved by the local medical-ethical committee, and all participants gave their informed consent.

Processing and Treatments of Human Ejaculated Sperm

For each experiment the ejaculates of three different normozoospermic healthy donors or four from varicocele donors were pooled and processed as previously described [24]. The same number for both normal and pathological samples of Percoll-purified sperm was washed with unsupplemented Earle's medium (uncapacitating medium) and resuspended in the same medium.

Western Blot Analysis of Sperm Proteins

Each sperm sample was centrifuged for 5 min at 5000*g*. The pellet was resuspended in lysis buffer as previously described [25]. An equal amount of protein (80 μg) was boiled for 5 min, separated on a 11% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with an appropriate dilution of the indicated primary Ab. The binding of the secondary Ab was revealed with the ECL Plus western blotting detection system, according to the manufacturer's instructions.

Transmission Electron Microscopy (TEM) with Immunogold Analysis for PI3K

The method was performed as described with minor modifications [26]. Sperm fixed overnight in 4%

paraformaldehyde were washed in phosphate-buffered saline (PBS) to remove excess fixative, dehydrated in graded alcohol, infiltrated in LR white resin, and polymerized in a vacuum oven at 45 °C for 48 h. Ultrathin sections (60 nm) were cut and placed on coated nickel grids for postembedding immunogold labeling with the rabbit polyclonal Ab to human PI3K. Potential nonspecific labeling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% bovine serum albumin (BSA), and 0.1% coldwater fish gelatin at room temperature for 1 h. Sections were then incubated overnight at 4 °C with rabbit polyclonal PI3K Ab, at a dilution of 1:500 in PBS buffer. Afterward, the grids were washed rigorously several times with drops of PBS + 0.1% BSA, and incubated with 10-nm γ-globulin goat anti-rabbit–gold particle complex at a dilution of 1:50 for 2 h at room temperature. The sections were then washed in PBS, later fixed in glutaraldehyde, counterstained in uranyl acetate and lead acetate, and examined under a Zeiss EM 900 TEM. To assess the specificity of the immunolabeling, negative controls were carried out in corresponding sections of sperm that were labeled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of the primary Ab.

Statistical Analysis

At least four independent experiments were performed.

RESULTS

PI3K Is Expressed in Normal and Varicocele Sperm Samples

First we investigated the expression of PI3K in normal (N) and varicocele (V) sperm samples by Western blotting analysis. As shown in Figure 1(a), one PI3K immunoreactive band was detected in normal samples at the expected size of 110 kDa, showing similar molecular weight and expression level observed in T47D and MCF-7 breast cancer cells, used as positive control [27]. Surprisingly, in varicocele samples we evidenced a reduced expression of PI3K. When we compared PI3K expression among normal, OAT, and varicocele spermatozoa, only the latter showed a marked decrement, addressing a role for PI3K in varicocele pathophysiology (Figure 1b).

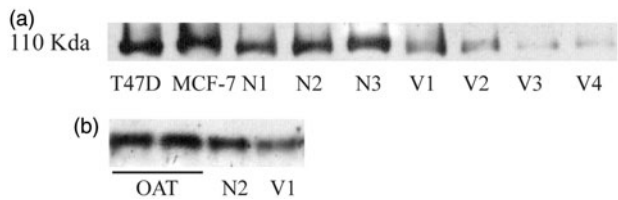

FIGURE 1. Western blot analysis of human sperm proteins showed expression of the conventional PI3K (p110). (a) N1, N2, N3 pooled samples of ejaculated spermatozoa from healthy donors. V1, V2, V3, V4 pooled samples of ejaculated spermatozoa from varicocele donors. T47D and MCF-7 extracts were used as controls. (b) OAT pooled samples of ejaculated spermatozoa from oligoasthenoteratozoospermic patients. The number on the left indicates the molecular masses (kilodaltons) of the marker proteins. The experiments were repeated at least three times and images show the results of one representative experiment.

Ultrastructural PI3K Expression in Human "Healthy" Sperm Samples

To investigate subcellular distribution of PI3K protein we performed an ultrastructural analysis of spermatozoa by TEM with immunogold analysis. Our results confirmed that PI3K was expressed in normal human sperm. As shown in Figure 2, spermatozoa from healthy donors showed clearly identifiable immunoreactions. Interestingly, the label decorated mostly the head at both the membrane and nucleus levels (Figure 2a) and the entire tail, from the middle piece (Figure 2b) to the end piece (Figure 2c).

In the sperm head, gold particles marking PI3K were mainly present on the apical region of the acrosome and in the nucleus and in the neck, while no appreciable labeling was detected over the post-acrosomal area region. Specifically the neck and the midpiece with the mitochondria also showed an appreciable presence of gold particles (Figure 2a, A). In the midpiece of the sperm tail (Figure 2b), label for PI3K was found in the axoneme (Figure 2b, A) in the swollen space between the mitochondria and only occasionally in association with the outer mitochondrial membrane. Positive signals were also present between the ribs of the fibrous sheet both in the middle and the principal piece of the tail, although it was progressively reduced from the principal piece up to the end piece (Figure 2c). All corresponding sections treated with BSA/PBS instead of primary antibodies, which served as negative controls, were free of labeling.

Ultrastructural PI3K Expression in "Varicocele" Sperm

In "varicocele" sperm we observed a decrease of PI3K (Figure 3) expression with respect to the healthy samples. PI3K label appears to be confined to the head, since it was not detectable in the neck or in the midpiece (Figure 3a, b). In the head, immunogold reactions were localized both at the plasma membrane and in the nucleus. A strong reduction in gold particles was evidenced in the neck and along the midpiece and the tail (Figure 3b, c) of all the varicocele

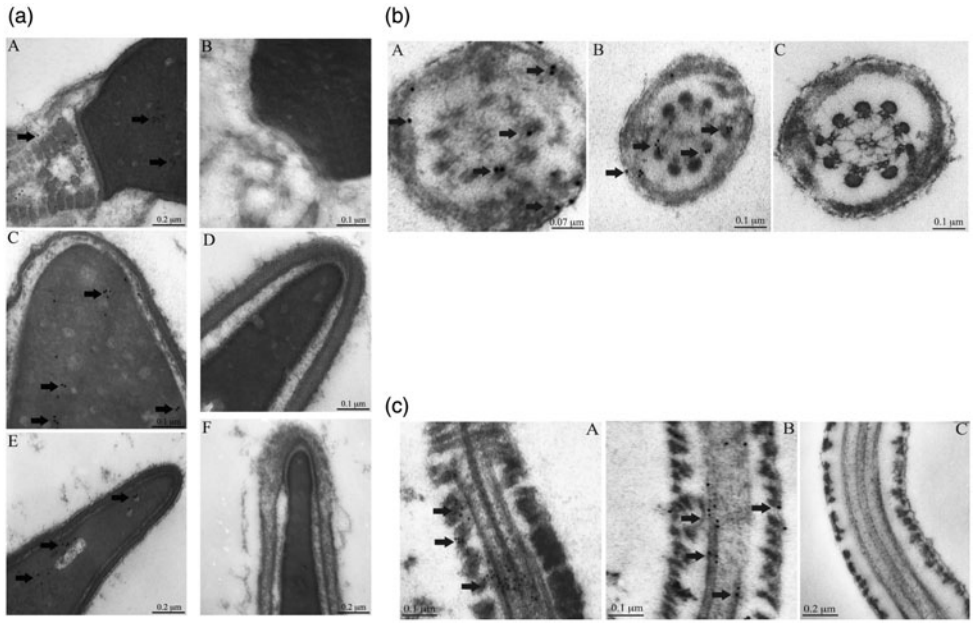

FIGURE 2. Immunoelectron microscopic localization of PI3K in normal human spermatozoa. Sperm were collected and prepared as described under Materials and Methods. Micrographs of sections from ejaculated sperm of normal patients probed with rabbit polyclonal Ab to human PI3K. In all cases, a secondary anti-rabbit antibody conjugated to 10-nm colloidal gold particles was used for labeling. (a) Sections through the head; (b, c) cross sections and longitudinal sections of the midpiece of the flagellum. Pictures are representative of three similar experiments. Scale bars are indicated. Negative controls are carried out in corresponding sections of spermatozoa that were labeled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of primary Ab.

spermatozoa, as we can observe in a longitudinal section of the principal piece (Figure 3c). Negative controls were almost completely free of gold-sphere labeling.

DISCUSSION

Varicocele may be responsible for nearly one-third of cases of male infertility. The idea that varicocele plays a detrimental role in fertility is supported by the presence of a higher frequency of affected men among the infertile population (25.4%) than among men with normal semen parameters (11.7%) [2]. Many efforts have been made to find semen indicators of varicocele; nonetheless, its management continues to stimulate controversy among reproductive experts. Few recent studies have reported molecular alterations related to varicocele. Decreased E2/ER signaling [28], reduced PR levels [24], or altered expression pattern and subcellular localization of COX-1 and COX-2 [23] were shown to be detrimental effects at molecular level on varicocele spermatozoa. These alterations may in turn negatively influence male reproduction, although the exact molecular mechanisms are still not fully clarified. Therefore, their definition is desirable for development of novel therapeutic or preventive strategies.

Our recent studies strongly suggest a role for PI3K as mediator of steroid receptors signaling influencing sperm metabolism and motility [17,18] and it was reported that PI3K also mediates actin polymerization during sperm capacitation [10]. In the present study we evidence the expression levels of PI3K protein and the subcellular distribution in human ''healthy'' spermatozoa and varicocele samples. Interestingly, we highlight a difference in PI3K location other than its reduced levels in ''varicocele'' sperm. PI3K analyzed by Western blotting was readily detectable in the protein lysates of human healthy ejaculated spermatozoa at the same apparent molecular weight as the stained band of MCF7 and T47D cells used as positive control cells [27].

To the best of our knowledge, the present study is the first in which TEM immunogold analysis was applied to analyze PI3K subcellular distribution in sperm samples from healthy volunteer donors of proven fertility and varicocele patients. Previous studies evidenced the presence of both catalytic and regulatory subunits of the enzyme in human spermatozoa by immunoprecipitation, western analysis, and immunofluorescence microscopy [9].

Our results demonstrate by TEM the specific localization of PI3K in healthy human sperm and the decrease in the amounts and the different sperm cell distribution of the enzyme in the pathological samples. The immunocytochemical data corroborate the findings of western blot analysis, demonstrating decreased expression of PI3K in varicocele samples. It was reported that proteins such as

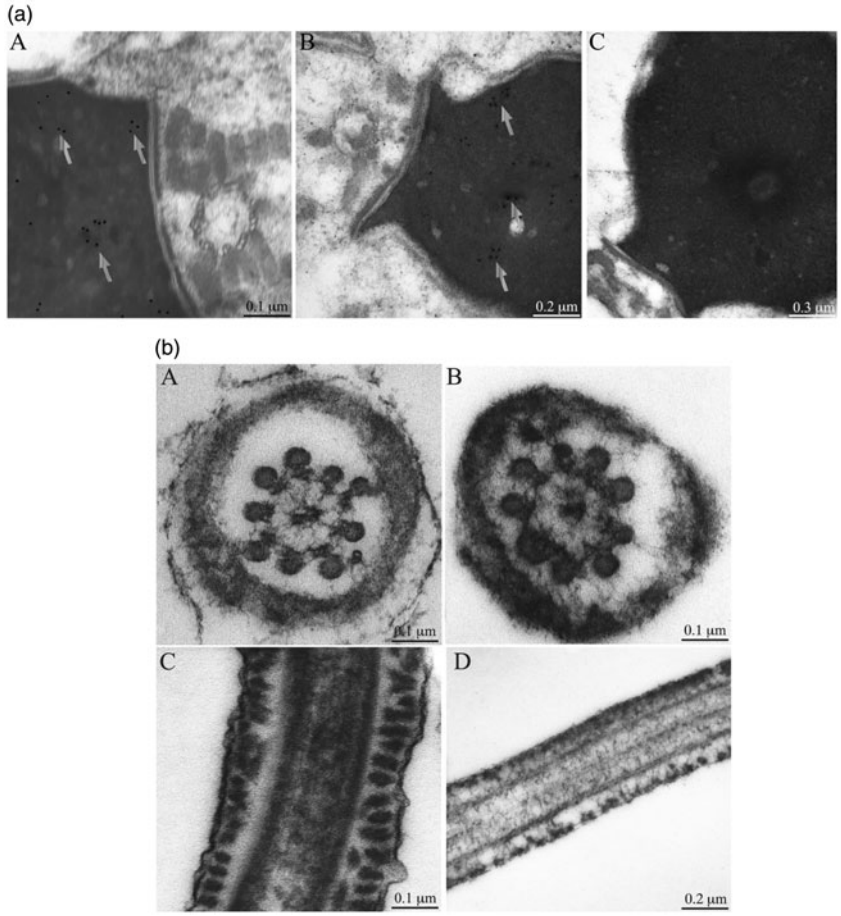

FIGURE 3. Immunoelectron microscopic localization of PI3K in varicocele spermatozoa. Sperm were collected and prepared as described under Materials and Methods. Micrographs of sections from ejaculated sperm of varicocele patients probed with rabbit polyclonal Ab to human PI3K. In all cases, a secondary anti-rabbit antibody conjugated to 10-nm colloidal gold particles was used for labeling. (a) Sections through the head; (b, c) longitudinal sections and cross sections of the midpiece of the flagellum. Pictures are representative of three similar experiments. Scale bars are indicated. Negative controls are carried out in corresponding sections of spermatozoa that were labeled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of primary Ab.

glucocorticoid receptor are rapidly degraded in heat-shocked cells [29]; therefore, it may be possible that the increased testicular temperature in varicocele subjects determines this effect.

In ''healthy'' sperm, we localized the PI3K protein specifically in the head at both the membrane and nucleus levels and the entire tail, from the middle piece to the end piece, evidencing numerous gold particles. These results are partially in agreement with recent immunocytochemical studies [30] evidencing elements of the PI3K signaling complex localized to relative domains in human spermatozoa. This evidence is in agreement with our data, which suggested that the enzymes are located in the principal piece of the sperm tail, the neck, and the acrosome.

The lower expression and altered subcellular location of PI3K in ''varicocele'' sperm compared with healthy samples may happen during spermatogenesis when factors specifically expressed could interfere with the expression of the PI3K, or during spermiogenesis when the formation of the flagellum occurs. The varicocele-induced damage may also happen during the transit of the sperm along the male reproductive tract (epididymus and sex accessory glands), where it undergoes modifications in macromolecule composition.

Our results acquire more emphasis since they evidence important alterations regarding the PI3K expression in varicocele sperm samples. We show a strong decrease of the PI3K in this disease as demonstrated by our western blotting analysis on varicocele sperm. Furthermore, a strong reduction in gold particles was evidenced in the neck and along the midpiece and the tail of all sperm varicocele tested; thus, PI3K label appears to be confined to the head. The molecular damage may explain reduced fertilization potential since varicocele sperm may be less responsive to factors such as PI3K that influence capacitation process and acrosome reaction.

Recent studies demonstrated the presence of PIK3R1 (p85 regulatory subunit of PI3K) in bovine

sperm, which is localized to the sperm midpiece and the postacrosomal region of the head [10] and also in boar, hamster, and mouse. It was strongly suggested that it is involved in sperm capacitation and the acrosome reaction [9,31–34]. Furthermore, the PI3K regulatory subunit p85 was shown to be gradually phosphorylated during mouse sperm capacitation [35]. It has been suggested that sperm enkurin may tether p85 to a ZP3-activated TRPC cation channel [36], indicating a possible involvement of PI3K in the acrosome reaction. Specifically, sperm interaction with the zona pellucida results in ZP3 activation of TRPC channels, leading to both Ca^{2+} entry and to activation of PI3 kinase. The resulting D3 phosphorylation of PIP2 (phosphatidylinositol-4,5-bisphosphate) leads to the local accumulation of PIP3 (phosphatidylinositol-3,4-5-triphosphate), resulting in the activation of the serine/theonine protein kinases Akt (protein kinase B) and PKCζ. These protein kinases mediate the downstream stages of sperm exocytosis [21,37].

Our previous data strongly support the role of PI3K in cell survival [19]. In fact, we have demonstrated that the estradiol-induced effects on sperm are mediated by an enhanced phosphorylation of the proteins involved in the PI3K/Akt pathway [16]. In uncapacitated sperm, both insulin and leptin increased PI3K activity, modulating sperm capacitation and metabolism [17,18].

Our present report correlates well with these data, and the reduced expression of PI3K in varicocele may partially explain the reduced fertilization potential in this disease. In conclusion, our data demonstrating differences of PI3K subcellular localization in a highly polarized cell such as a spermatozoan suggest how this could influence compartmentalized-specific signaling pathways to determine molecular basis of reduced fertility in varicocele. The marked reduced expression of PI3K in the flagellum and in the head of varicocele sperm may explain the detrimental effects in this condition.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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The estrogen receptor α is the key regulator of the bifunctional role of FoxO3a transcription factor in breast cancer motility and invasiveness

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Abbreviations: Cav1, caveolin-1; E2, 17β-estradiol; ERα–, estrogen receptor alpha negative; ERα+, estrogen receptor alpha positive; FoxO3a, Forkhead box class O 3a; F3a, 1038 pcDNA3 flag FKHRL1 (Addgene) encoding full-length FoxO3a; F3aAAA, 1319 pcDNA3 flag FKHRL1 AAA (Addgene) encoding the constitutively active triple mutant of FoxO3a; IDC, invading ductal carcinomas; IHC, immunohistochemistry; DCIS, ductal carcinomas in situ; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKB, protein kinase B; PRF-CT, phenol red-free medium containing charcoal-treated FBS; PRF-GM, PRF-growing medium; PRF-SFM, PRF and serum-free media; siCav1, siRNA for effective depletion of Caveolin-1 transcripts; siER, siRNA for effective depletion of ERα transcripts; siF3a, siRNA for effective depletion of FoxO3a transcripts; WB, western blotting assay

The role of the Forkhead box class O (FoxO)3a transcription factor in breast cancer migration and invasion is controversial. Here we show that FoxO3a overexpression decreases motility, invasiveness, and anchorage-independent growth in estrogen receptor α-positive (ERα+) cancer cells while eliciting opposite effects in ERα-silenced cells and in ERα-negative (ERα–) cell lines, demonstrating that the nuclear receptor represents a crucial switch in FoxO3a control of breast cancer cell aggressiveness. In ERα+ cells, FoxO3a-mediated events were paralleled by a significant induction of Caveolin-1 (Cav1), an essential constituent of caveolae negatively associated to tumor invasion and metastasis. Cav1 induction occurs at the transcriptional level through FoxO3a binding to a Forkhead responsive core sequence located at position –305/–299 of the Cav1 promoter. 17β-estradiol (E2) strongly emphasized FoxO3a effects on cell migration and invasion, while ERα and Cav1 silencing were able to reverse them, demonstrating that both proteins are pivotal mediators of these FoxO3a controlled processes. In vivo, an immunohistochemical analysis on tissue sections from patients with ERα+ or ERα– invasive breast cancers or in situ ductal carcinoma showed that nuclear FoxO3a inversely (ERα+) or directly (ERα–) correlated with the invasive phenotype of breast tumors. In conclusion, FoxO3a role in breast cancer motility and invasion depends on ERα status, disclosing a novel aspect of the well-established FoxO3a/ERα interplay. Therefore FoxO3a might become a pursuable target to be suitably exploited in combination therapies either in ERα+ or ERα– breast tumors.

Introduction

The forkhead box class O3a (FoxO3a) is one of the four members (FoxO1a, FoxO3a, FoxO4, and FoxO6) belonging to the subfamily of winged-helix forkhead transcription factors (FoxOs), whose functions are negatively regulated by the insulin-phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB) signaling.[1] In the absence of insulin or growth factors, FoxOs are mainly located within the nuclei and regulate a set of target genes, thereby promoting cell cycle arrest, stress resistance,

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apoptosis, DNA damage repair, and metabolism.[2] In presence of insulin or growth factors, FoxOs undergo phosphorylation, bind to the chaperone proteins 14-3-3 and are exported into the cytoplasm, where they are degraded via the ubiquitin–proteasome pathway.[1]

An increasing interest in FoxOs factors has been lately observed in the oncologic research field. In particular, in breast cancer, its role is still controversial, in fact, FoxO3a overexpression has been shown to inhibit tumor growth in vitro and tumor size in vivo,[3-5] and cytoplasmic location of FoxO3a seems to correlate with patients poor survival.[3] Moreover, genetic deletion of the FoxOs alleles (FoxO1a, FoxO3a, and FoxO4) generates progressive cancerous phenotypes, such as thymic lymphomas and hemangiomas. These data elucidate FoxOs as bona fide tumor suppressor genes.[6] Additionally, FoxO members seem to be important mediators of the well-established functional cross-talk between estrogens and growth factors, which play a pivotal role in breast cancer development and progression.[7] In fact, growth factors are known to influence the expression and activity of estrogen receptor α (ERα) and its transcriptional cofactors; conversely, ERα regulates the expression of growth factor receptors and their ligands and signaling intermediates.[8] In this context, several reports have recently suggested a functional interaction between ERα and FoxO members. 17β-estradiol (E2) has been noted to determine ERα binding to FoxO1a, FoxO3a, and FoxO4, which, in turn, showed either coactivator or corepressor functions on estrogen-responsive element (ERE) sites, depending on the cellular model.[5,9,10] Moreover, we introduced the importance of Akt2/FoxO3a axis in the control of ERα-mediated transcription in ERα-positive (ERα+) breast cancer cells. Our results indicate that Akt2 inhibition reduces ERα transcriptional activity through FoxO3a activation, suggesting that FoxO3a, acting as a co-repressor for ERα, could exert a protective role in ERα+ breast tumors.[11]

In line with this assumption, Belguise et al. showed that ectopic expression of a constitutively active FoxO3a overrode transforming growth factor-B1-mediated invasive phenotype and induced a more epithelial phenotype in ERα+ mouse mammary tumors.[12] However, more recently, FoxO3a has been described to behave in an opposite fashion in several other cancer cell lines, which, interestingly, were all ERα-negative (ERα-); in fact, Storz et al. reported that, in tested cells, nuclear retention of FoxO3a resulted in greatly increased invasion, through the induction of matrix metalloproteinase 9 (MMP-9) and MMP-13.[13] Due to the inconsistency of the data available from ERα+ and ERα– breast cancer cells, the interplay between ERα and FoxO3a in tumor metastasis needs further investigations and is the goal of the present study. Since it is well documented that, in breast cancer, ERα signaling strongly correlates with a lower invasiveness and reduced metastatic potential,[14] we assume that FoxO3a/ERα interplay could be responsible for the reduction of the migrating and invasive phenotype only in ERα+ cells, while, in ERα– cells, the lack of the α isoform of the receptor might enable FoxO3a to act in an opposite fashion. Thus, the present work was aimed to undertake an accurate study on the molecular mechanisms through which FoxO3a regulates migration and invasion in ERα+ breast cancer cells. Our results offer new interesting insights on FoxO3a activity, elucidating additional mechanisms that could represent novel targets in breast cancer therapy.

Results

Cell motility, invasion, and anchorage-independent growth are inhibited in ERα+ breast cancer cells overexpressing FoxO3a

To assess the role of FoxO3a in the metastatic and invading potential of breast cancer cells, wild-type FoxO3a (F3a) was overexpressed in ERα+ MCF-7. Our results show a significant reduction of migrating and invading MCF-7/F3a cells (**Fig. 1A and B**), compared with control samples. Ectopic expression of the constitutively active triple mutant of FoxO3a (F3aAAA), where the 3 known PKB phosphorylation sites have been mutated to alanine, so that FoxO3a can no longer be inhibited by PKB-mediated phosphorylation, emphasized the phenomenon (**Fig. 1A and B**), suggesting that FoxO3a modulation of the migrating and the invading potential could involve the transcriptional induction of Forkhead responsive genes. FoxO3a silencing (siF3a) confirmed these data, since it led to a substantial increase in cell migration and invasion (**Fig. 1A and B**). Moreover, in agreement with our previous observations,[15] E2 treatment strongly reduced motility and invasion, and the effect was additive in F3a- and F3aAAA-overexpressing samples, while siF3a only in part was able to counteract E2-mediated effects (**Fig. 1A and B**).

In addition, anchorage independence, a characteristic of malignancy and tumor progression, was also investigated in F3a-overexpressing and silenced MCF-7 cells through soft agar colony-formation assay. We observed a dramatic decrease of the number as well as of the dimensions of the colonies in MCF-7/F3a samples, reaching almost completely the condition of single cells in F3aAAA-expressing cells (**Fig. 1C$_1$ and C$_2$**). The same trend was evidenced in E2-treated samples, showing how FoxO3a, especially in its active form, is able to counteract the well-known positive effect of the nuclear hormone on the colony formation of MCF-7 cells.[16] As expected, an increase in the number of colonies was observed following siF3a, and such increase became more evident in presence of E2 (**Fig. 1C$_1$ and C$_2$**). Transfections and silencing efficiency were assessed on total protein lysates (**Fig. 1D**).

Interestingly, F3a and F3aAAA overexpression in other ERα-positive cell lines, ZR-75 (breast cancer) and Ishikawa (endometrial cancer), led to results that were comparable to those obtained from MCF-7, both in presence or absence of E2 (**Fig. S1**, upper panels)

The lack of ERα reverses FoxO3a-mediated inhibition of migration, invasion, and colonies formation

To assess if the effects of FoxO3a on motility, invasiveness, and colony formation could depend on ERα, silencing experiments were conducted in MCF-7, using specific siRNAs against ERα (siER) (**Fig. 2**). Interestingly, ERα silencing was able to counteract FoxO3a-mediated inhibition of the above-mentioned pathological features.

In particular, compared with control (siScramble), siER led to an increase in cell migration and invasion, which became even

more evident in F3a and, especially, in F3aAAA-expressing cells (**Fig. 2A and B**), confirming that ERα is a hallmark of a less motile and invading phenotype,[15,17] and that FoxO3a's effect on cell motility and invasiveness can switch from inhibitory to stimulatory, depending on the presence or absence of ERα, respectively. Moreover, in siER samples, reasonably due to the lack of the receptor, E2 treatment no longer caused the reduction of the invading potential of MCF-7 (**Fig. 2B**) and even showed the opposite effect on cell motility, which rather increased over the respective controls (**Fig. 2A**). These evidences suggest that, in absence of a functional ERα, E2 could trigger some other pathway that stimulates cell migration (although not invasion), and that FoxO3a can somehow cooperate with the hormone in this process.

As expected, ERα silencing was able to inhibit both basal and E2 induced MCF-7 growth in soft agar by strongly reducing the number and the dimensions of colonies compared with non-treated and E2-treated siScramble samples, respectively

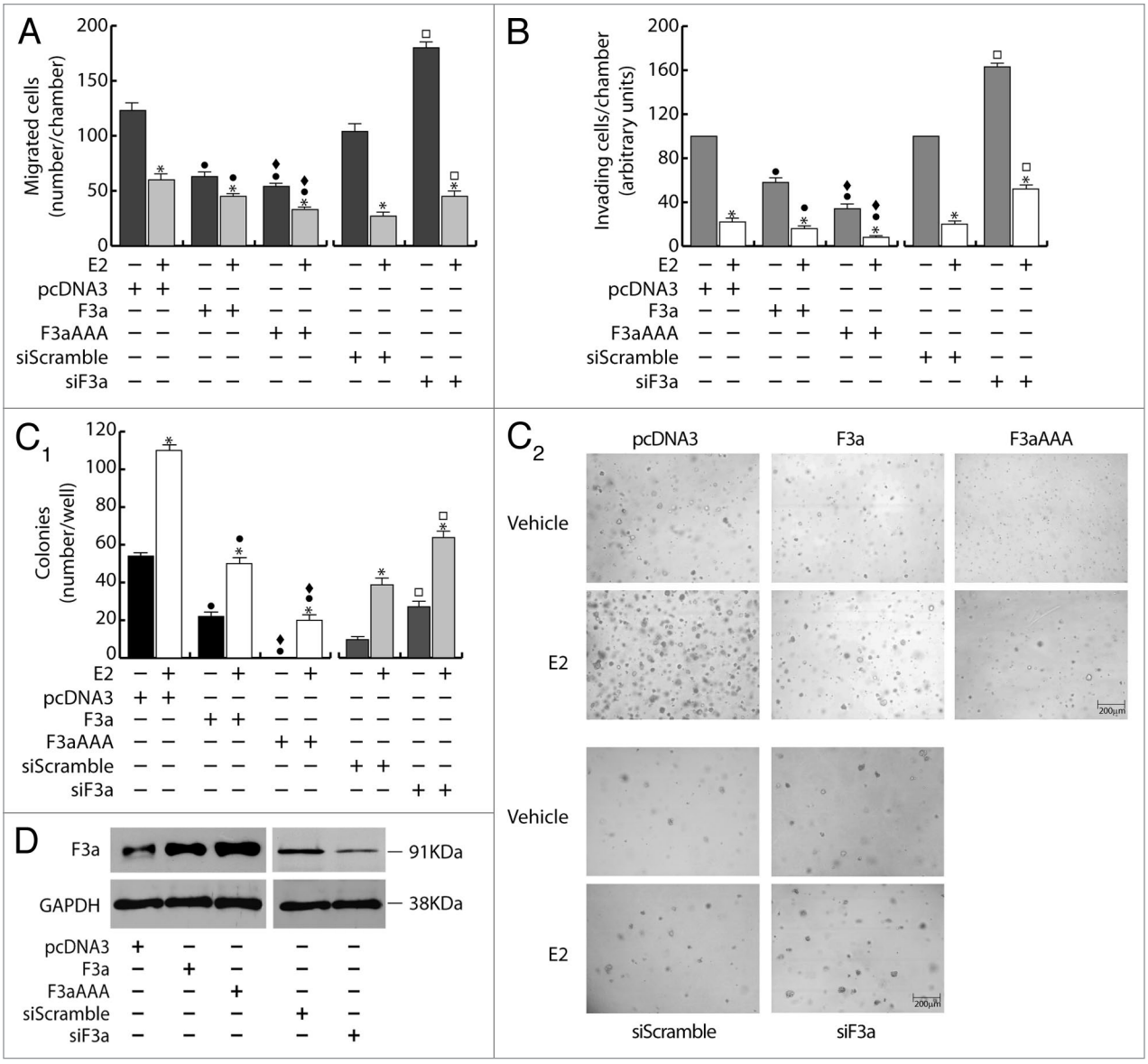


Figure 1. FoxO3a inhibits migration, invasion and anchorage independent growth in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells was transiently transfected with 1 μg/35 mm dish of F3a, F3aAAA, or pcDNA3 as control. Another double set was silenced for FoxO3a expression (siF3a), using a siScramble as control (60 pmol siRNAs/35 mm dish). After 5 h cells were switched to PRF-SFM, and the next day one of each set of cells was harvested and subjected to migration (**A**), invasion (**B**), and soft agar assay (**C_1 and C_2**). Migration and invasion assays were conducted as described in "Materials and Methods", adding 100 nM E2 in the bottom of the wells where indicated. Migrated and invading cells were evaluated after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies >50 μm diameter formed after 14 d from plating were photographed at 4× magnification (**C_2**) and counted under the microscope (**C_1**). The second set of either transfected or silenced MCF-7 cells was used for total protein extractions and WB analysis to assess transfections efficiency; GAPDH was evaluated as a loading control (**D**). Results are reported as the mean ± s.d. of at least 3 independent experiments. In all experiments, significance values were as follows: *, $P < 0.01$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦, $P < 0.05$ vs. corresponding F3a; □ , $P < 0.01$ vs. corresponding siScramble.

(**Fig. 2C**). However, as in migration and invasion experiments, the inactivation of the nuclear receptor reversed the effect of ectopic F3a and F3aAAA, which, either in absence or presence of E2 treatment, induced an increase in the number of colonies, instead of the decrease observed in siScramble samples (**Fig. 3C**).

The fact that ERα exerts a pivotal role in determining FoxO3a behavior was confirmed by the results obtained in ERα– cells. Indeed, overexpression of FoxO3a in ERα– breast cancer MDA-MB-231 cells was able to induce an evident increase (rather than a decrease, as in ERα+ cells) of the migrating and invading potential (**Fig. 3A and B**), as well as, when grown in soft agar, F3a-overexpressing cells formed many more and larger colonies compared with control vector (**Fig. 3C$_1$ and C$_2$**). Once again, in all experiments, F3aAAA was more effective than F3a, while an evident reduction of migration, invasion and number and dimensions of colonies was observed in F3a silenced samples (**Fig. 3A–C$_2$**). Transfections and silencing efficiency were determined concomitantly (**Fig. 3D**).

Noteworthy, as in MDA-MB-231, F3a and F3aAAA overexpression led to comparable results in other ERα– breast cancer cell lines (MDA-MB-468 and MDA-MB-435) as well as in ERα– cervical cancer HeLa cells, indicating that FoxO3a functions trough mechanisms that are not tissue-specific (**Fig. S1**, lower panels and data not shown).

FoxO3a and E2 synergistically induce caveolin-1 expression in ERα+ cancer cells

To the aim of identifying the mechanism through which FoxO3a modulates cell motility and invasiveness, we focused our attention on caveolin-1 (Cav1), a protein that has been reported to be induced by both Forkhead transcription factors[18] and E2.[19,20] Since, in breast cancer, Cav1 has been negatively[21] and positively[22] linked to tumor progression, motility, and invasiveness, we questioned if FoxO3a could control migration and invasion of breast cancer cells through the modulation of Cav1 expression.

In ERα+ MCF-7 cells, the ectopic expression of FoxO3a caused a strong upregulation of Cav1 protein and mRNA, which

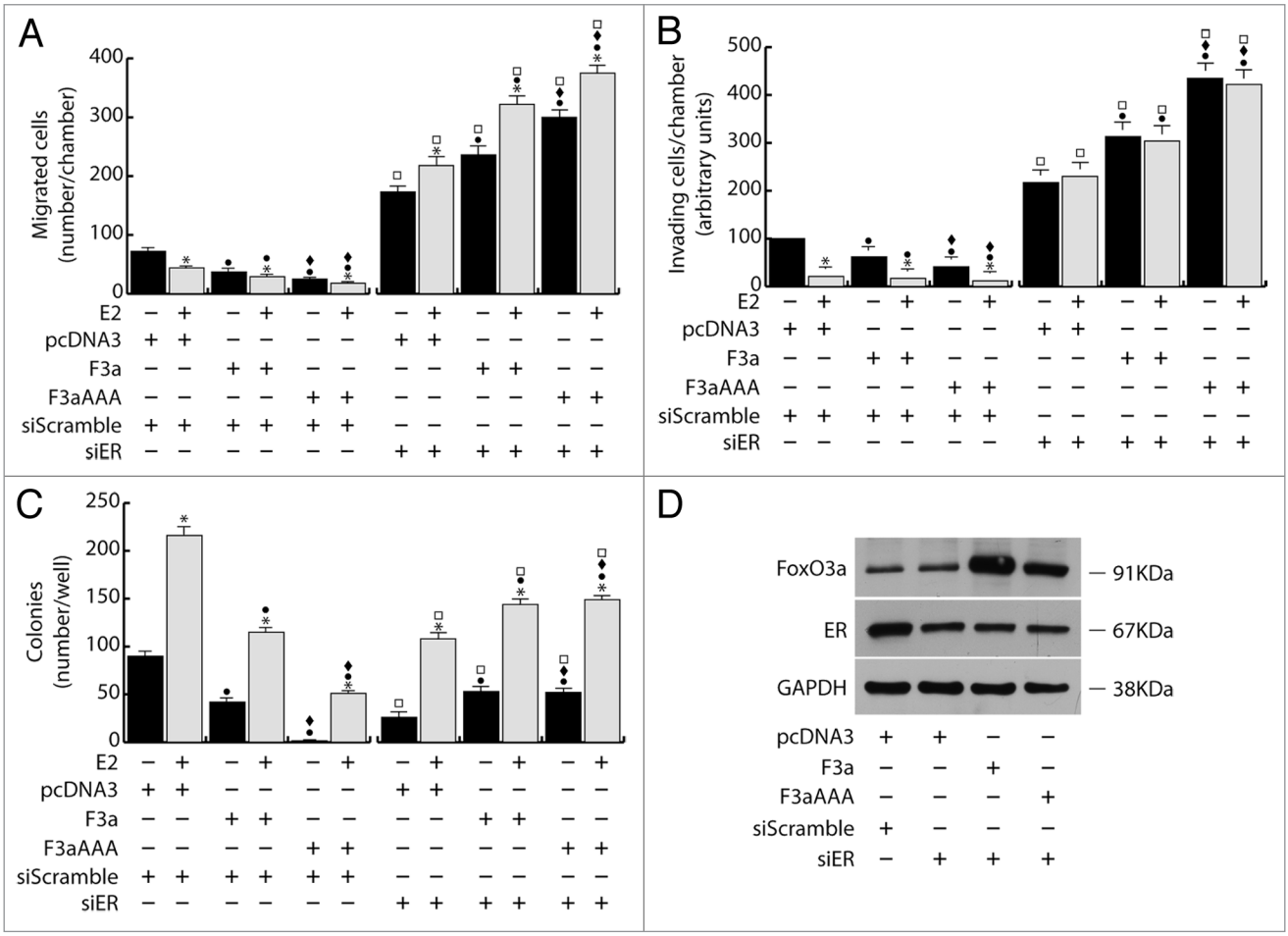


Figure 2. FoxO3a mediated inhibition of breast cancer cell migration, invasion and growth in suspension depends on ERα Two double sets of MCF-7 cells were silenced either for ERα (siER), using siScramble as control. After 5 h cells were switched to PRF-SFM and transiently transfected with F3a, F3aAAA, or pcDNA3. Next day cells were harvested and one set of each experiment was subjected to migration, invasion, and soft agar assay in the presence or in the absence of E2. Migrated (**A**) and invading (**B**) cells were evaluated after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies ≥50 μm diameter formed after 14 d from plating were counted under the microscope (**C**). The second set of each experiment was used for total protein extraction to evaluate transfections efficiency by WB analysis; GAPDH was used as loading control (**D**). Results are the mean ± s.d. of at least three independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦, $P < 0.01$ vs. corresponding F3a; □ , $P < 0.01$ vs. corresponding siScramble.

was even more evident in F3aAAA transfectants, suggesting that FoxO3a induction of Cav1 expression could occur at the transcriptional level. As expected, E2 treatment increased Cav1 levels, and the effect was additive to that exerted by F3a or F3aAAA (**Fig. 4A and B**). Silencing experiments confirmed FoxO3a involvement in Cav1 transcription, leading to a decrease in Cav1 content and attenuating the E2-dependent Cav1 induction (**Fig. 4C and D**). Notably, Cav1 undergoes similar regulation by E2 and FoxO3a in the other 2 tested ERα+ cell lines, ZR-75 and Ishikawa (**Fig. S2**). In particular, the induction of Cav1 by E2 is ERα-dependent, since (1) the pure antiestrogen ICI 172.780 was able to abrogate the effect of E2 on Cav1 expression in ERα+ MCF-7 cells (**Fig. 4E**); and (2) the hormone did not increase Cav1 expression in ERα–, although ERβ+, MDA-MB-231 cells (**Fig. 4F**).

In light of these evidences we could hypothesize that, in ERα+ cells, FoxO3a might promote a less aggressive phenotype by cooperating with the hormone receptor in *CAV1* gene induction.

Cav1 is a mediator of FoxO3a-dependent inhibition of migration, invasion, and growth in suspension in ERα+ breast cancer cells

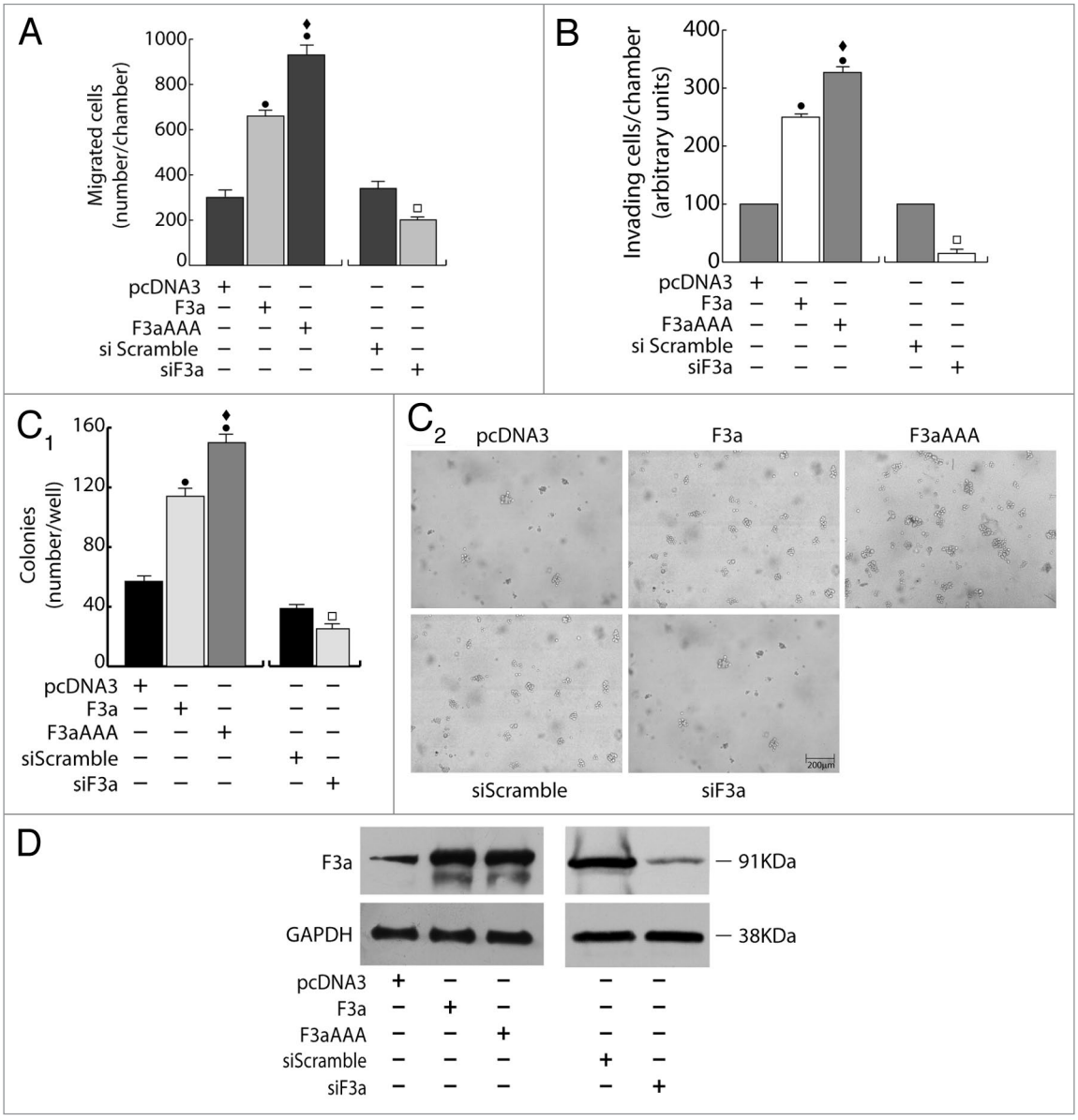


Figure 3. FoxO3a promotes migration, invasion, and anchorage-independent growth in ERα– MDA-MB-231 breast cancer cells. A double set of MDA-MB-231 cells were transiently transfected with 1 μg/35 mm dish of F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a expression (siF3a) using a siScramble as control (60 pmol siRNAs/35 mm dish). Both transfection and silencing were made on cells in suspended PRF-GM. After 5 h cells were serum starved and, 24 h later, harvested. One set was subjected to migration (**A**), invasion (**B**), or soft agar assay (**C_1 and C_2**). Migrated and invading cells were evaluated after 16 h and 48 h of incubation, respectively. In soft agar assay, colonies > 50 μm diameter formed after 14 d from plating were photographed at 4× magnification (**C_2**) and counted under the microscope (**C_1**). The second set of either transfected or silenced MCF-7 cells was used to assess transfections efficiency by WB analysis on total protein extracts; GAPDH was evaluated as a loading control (**D**). Results are reported as the mean ± s.d. of at least 3 independent experiments. ●, $P < 0.01$ vs. pcDNA3; ♦, $P < 0.01$ vs. F3a; □ , $P < 0.05$ vs. siScramble.

Cav1 involvement in FoxO3a-mediated inhibition of motility, invasiveness, and colonies formation was assessed by silencing experiments using specific siRNAs against Cav1 (siCav1) in ERα+ breast cancer cells, (**Fig. 5A–D**). Cav1 silencing was able to counteract FoxO3a effects, leading to an overall increase of cell migration and invasion in MCF-7 cells, although F3a and F3aAAA overexpression did not contribute to such increase, nor was siCav1 sufficient to completely reverse the inhibitory effect exerted by E2 treatment (**Fig. 5A and B**). A similar trend was observed in soft agar experiments, where the number of colonies was much greater in siCav1 samples, especially under E2 treatment (note that ERα protein content was not affected by siCav1, **Fig. 5D**), compared with the respective controls (siScramble) (**Fig. 5C**). Again, F3a and F3aAAA did not have any additive effect on colony growth (**Fig. 5C**).

These results show how, in MCF-7, FoxO3a control of cell migration, invasion, and anchorage-independent cell growth depends, in part, on Cav1, while it is strictly linked to ERα expression (**Fig. 2**). Indeed, in Cav1-negative T47D cells, which, in addition, bear a very low content of ERα, F3a, and F3aAAA overexpression did not lead to any significant decrease in motility, invading potential and colony formation in soft agar, reflecting a sort of compromise between the results observed following either Cav1 or ERα silencing in MCF-7 cells (**Figs. 2 and 5E–G**), thus indicating that these 2 proteins are mediators of both E2 and FoxO3a activity.

FoxO3a binds to and trans-activates the Cav1 promoter in MCF-7 cells

To deepen the understanding of the mechanism underlying the FoxO3a/ERα interplay in Cav1 induction, through an accurate analysis of the Cav1 promoter (GenBank accession #AF095591.1), we verified the presence of several Forkhead core sequences (FKHE), and we questioned if any of the identified regions may be involved in the FoxO3a/ERα-mediated

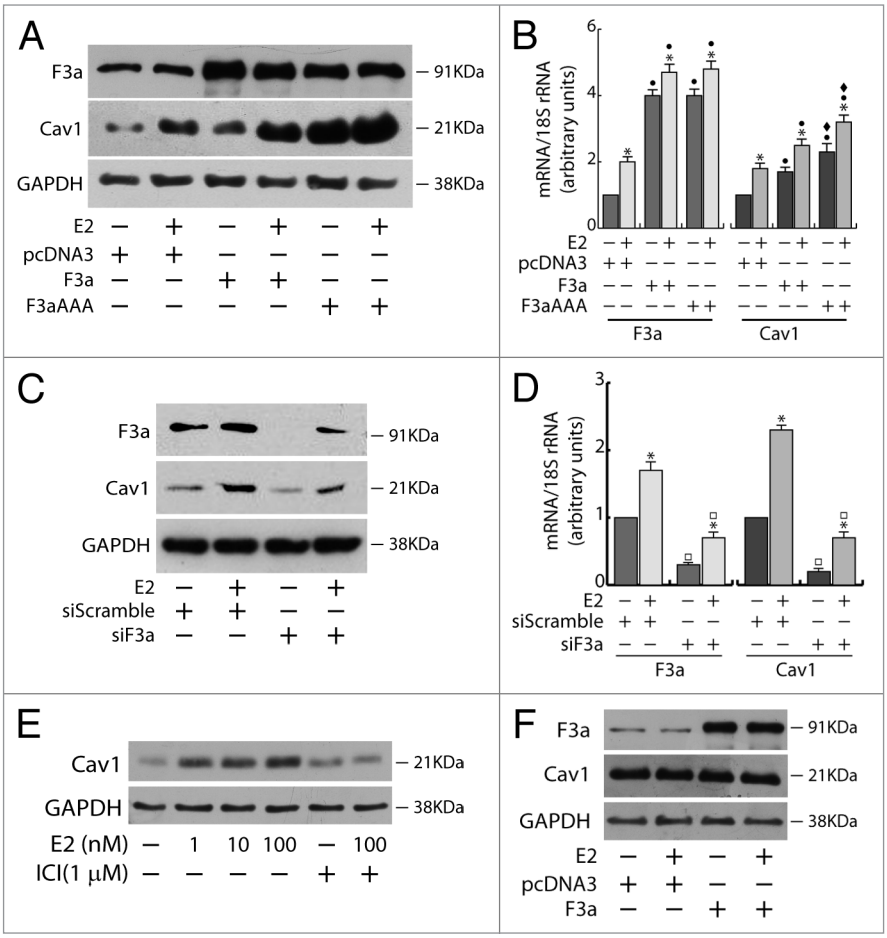


Figure 4. Cav1 expression depends on E2 and FoxO3a in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells were either transiently transfected with F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a, serum starved after 5 h and treated the next day with 100 nM E2 for 24 h. Cells were then harvested and total proteins and RNA were extracted, and subjected to WB (**A and C**) and RT-PCR analysis (**B and D**), respectively, for F3a and Cav1 expression assessment. (**E**) MCF-7 cells were seeded in growing medium, serum starved the next day for 24 h, pre-treated or not for 1 h with the pure antiestrogen ICI 182.780 and then treated with increasing concentrations of E2 (0, 1, 10, and 100 nM). (**F**) MDA-MB-231 cells were transiently transfected with F3a or pcDNA3 as control, serum starved for 24 h and then treated or not with 100 nM E2. After 24 h of E2 treatment, total proteins were extracted and subjected to WB analysis. GAPDH was analyzed as loading control in WB assays. For RT-PCR assays, each sample was normalized to its 18S rRNA content. Results are reported as the mean ± s.d. of at least 3 independent experiment. *, $P < 0.01$ vs. untreated; ●, $P < 0.01$ vs. pcDNA3; ♦, $P < 0.01$ vs. F3a; □ , $P < 0.05$ vs. siScramble.

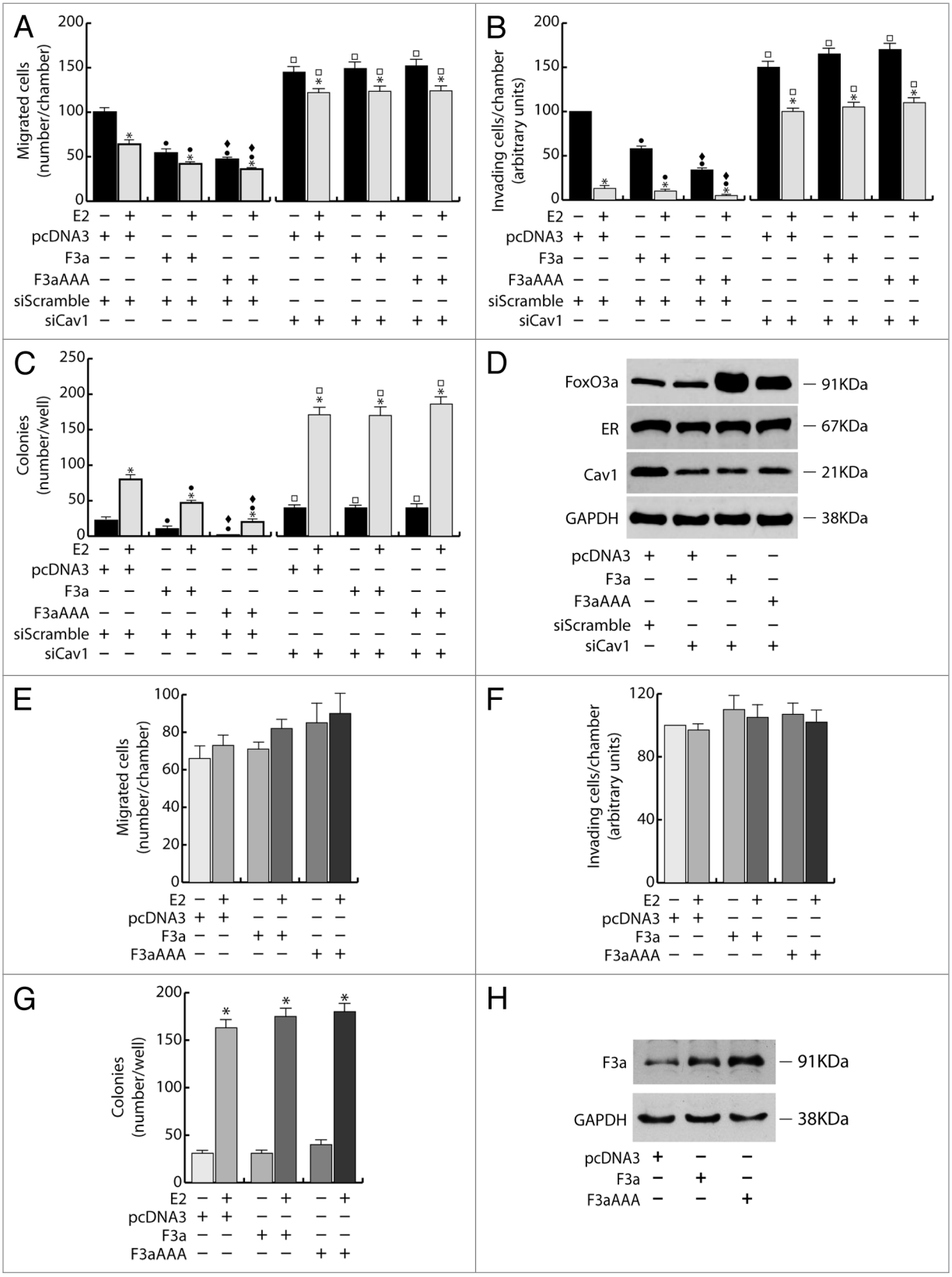


Figure 5. For figure legend, see page 3410.

Figure 5 (See previous page). Cav1 is a mediator of FoxO3a dependent inhibition of migration, invasion and growth in suspension of ERα+ breast cancer cells. (**A–D**) Two double sets of MCF-7 cells were silenced for Caveolin-1 (siCav1), using siScramble as control. After 5 h cells were switched to PRF-SFM and transiently transfected with F3a, F3aAAA, or pcDNA3. Next day cells were harvested and one set of each experiment was subjected to migration, invasion, and soft agar assay, in the presence or in the absence of E2. Migrated (**A**) and invading (**B**) cells were evaluated after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies ≥50 μm diameter formed after 14 d from plating were counted under the microscope (**C**). Transfection efficiency was evaluated by WB analysis on total protein extracted by the second set of cells; GAPDH was used as loading control (**D**). Results are the mean ± s.d. of at least 3 independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.01$ vs. corresponding pcDNA3; ♦, $P < 0.01$ vs. corresponding F3a; □ , $P < 0.01$ vs. corresponding siScramble. (**E–H**) A double set of T47D cells were transiently transfected with F3a, F3aAAA or pcDNA3. After 5h cells were switched to PRF-SFM and the next day one set of cells was harvested and subjected to migration (**E**), invasion (**F**), or soft agar assay (**G**), with or without 100 nM E2. Migrated and invading cells were counted after 24 h and 72 h of incubation, respectively. In soft agar assay, colonies formed after 14 d from plating were exposed to MTT and counted under the microscope. The second set of cells was lysed, and total protein was used for WB analysis to assess transfections efficiency; GAPDH was used as loading control (**H**). Results are the mean ± s.d. of at least 3 independent experiments. *, $P < 0.01$ vs. untreated.

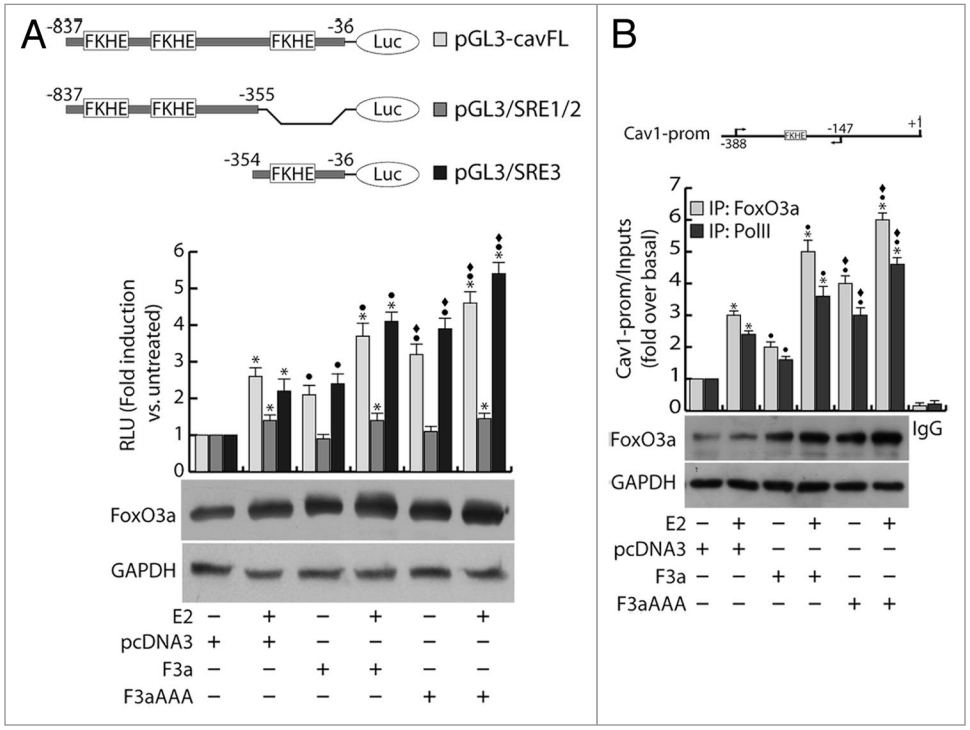


Figure 6. FoxO3a binds to and transactivates the Cav1 promoter. (**A**) MCF-7 were seeded in culture medium on 24-well plates, serum starved for 24 h, co-transfected in PRF-CT with pGL3-cavFL, or pGL3/SRE1/2, or pGL3/SRE3 and pRL-Tk, in presence of either pcDNA3 or F3a or F3aAAA vectors. After 6 h, E2 (100 nM) was added to the medium, where opportune, and the next day cells were harvested, and luciferase activity was evaluated. Cell extracts were also processed by WB analysis to assess F3a and F3aAAA transfection efficiency; GAPDH was used as loading control. (**B**) ChIP analysis was performed on the nuclear extracts from subconfluent MCF-7 cells seeded in 15 cm dish diameter, switched to PRF-SFM, and transfected with pcDNA3, F3a, or F3aAAA vectors. Twenty-four hours after transfection, the cells were treated with 100 nM E2 for 30 min or left untreated. The FKHE-containing Cav1 promoter region, precipitated with either anti-FoxO3a or anti-PolII pAbs were amplified using a specific pair of primers reported in "Materials and Methods". E2-treated samples were also precipitated with normal rabbit IgG and used as negative control. FoxO3a expression in transfected samples was analyzed by WB on Cytosolic lysates from the same set of cells. Data represents the mean ± s.d. of 3 independent experiments. *, $P < 0.05$ vs. untreated; ●, $P < 0.05$ vs. corresponding pcDNA3; ♦, $P < 0.05$ vs. corresponding F3a.

regulation of Cav1 gene expression in ERα+ breast cancer cells. To this aim, a vector bearing the luciferase gene under the control of the -837/-36 region of Cav1 promoter (pGL3-cavFL) was co-transfected with F3a or F3aAAA in MCF-7 cells and exposed or not to E2 treatment. In line with the results reported in **Figure 4A and B**, E2 stimulation significantly induced the Cav1 promoter activity, and such effect was increasingly higher in F3a- and F3aAAA-transfected cells (**Fig. 6A**). Interestingly, the construct pGL3/SRE1/2 (nt –837/–355), although containing FKHE core sequences, failed to be induced by FoxO3a but still weakly responded to hormone stimulation, most likely for the presence of Sp1 and AP-1 sites; on the contrary, the construct pGL3/SRE3 (nt –354/–36), bearing only one FKHE motif (nt –305/–299) and several Sp1 and AP-1 sites, was induced by both E2 and overexpressed FoxO3a, with a trend comparable to that observed with the pGL3-cavFL construct (**Fig. 6A**).

The involvement of E2 and FoxO3a in the transcriptional activation of the Cav1 promoter was corroborated by chromatin immunoprecipitation (ChIP) experiments, which evidenced a significant recruitment of FoxO3a on the region containing the –305/–299 FKHE sequence. Once again, E2 treatment strongly increased FoxO3a occupancy of the promoter, especially in

F3a- and F3aAAA-overexpressing samples (**Fig. 6B**). A similar pattern was observed in Polymerase II (PolII) precipitates, confirming that E2 and FoxO3a, both independently and synergistically, are able to induce Cav1 gene transcription (**Fig. 6B**).

Nuclear FoxO3a correlates in an opposite way with the tumor grade and the invasive phenotype in ERα+ and ERα– breast tumors

Tissue specimens from ductal carcinomas in situ (DCIS) and invading ductal carcinomas (IDC) (**Fig. 7J**) were analyzed to investigate if FoxO3a expression could correlate with the tumor grade and the invasive potential in ERα+ and ERα– breast tumors, as well as with Cav1 expression (in ERα+ tumors only).

In all sections, tumor cells were clearly distinguishable from either infiltrating immune cells or stromal cells. In non-invading, well-differentiated ERα+ tumors, FoxO3a was strongly expressed, showing a very high nuclear localization (**Fig. 7A**). Strikingly nuclear FoxO3a positivity was gradually lost in invading and less differentiated cells (see insets in **Fig. 7B**), while cytoplasmic localization was not as indicative. Concomitantly, Cav1 expression tended to decrease from tumors with positive to negative FoxO3a nuclear staining, and was completely lost in highly invading ERα+ tumors (**Fig. 7D–F**). Statistical analysis of these samples showed that both FoxO3a nuclear expression and Cav1 were inversely correlated with tumor grade and the invasive potential, while cytosolic FoxO3a did not result to be significantly correlated with any clinicopathological feature (**Fig. 7K**); moreover, Cav1 expression resulted directly correlated with FoxO3a nuclear content (**Fig. 7K**).

On the contrary, a very weak or even absent FoxO3a nuclear localization was observed in intraductal, well delimited areas of ERα– tumors (**Fig. 7G**), while a very strong nuclear staining was detected in invading areas of the same samples (**Fig. 7H**) and in clearly invasive carcinomas (**Fig. 7I**). This observation was confirmed by statistical analysis that evidenced a direct correlation between FoxO3a expression and both tumor grading and the invasive potential of ERα– breast cancer tissues (**Fig. 7L**).

Discussion

FoxO transcription factors are crucial for regulating a myriad of physiological processes, including proliferation, metabolism, cell differentiation, cell cycle arrest, DNA repair, and apoptosis. FoxOs also play important roles in tumorigenesis, since they have been shown to be deregulated in many types of human cancers, and restoring their expression/activity has been shown to be effective in tumor suppression.[2]

The involvement of FoxOs in tumor metastasis is controversial, e.g., FoxO3a has been reported to have either a protective or a promoting role on cell motility and invasion.[12,13] Our hypothesis was that such a difference might be ascribed to ERα status, since activated FoxO3a was able to reverse the invasive phenotype of ERα+ breast cancer cells[12] while promoting tumor cell invasion in other cancer cell lines, which, notably, were all ERα–.[13] Thus, the present study was aimed to verify if the effect exerted by FoxO3a on the metastatic potential of ERα+ breast cancer could derive from a general mechanism through which FoxO3a cooperates with the nuclear receptor in reducing motility and invasiveness of ERα+ tumors, while in absence of the receptor FoxO3a favors a more migrating and invasive phenotype. Indeed, since ERα signaling is well known to strongly correlate with a lower invasiveness and reduced motility of breast cancer cells,[15] and considering that increasing evidences recognize Forkhead factors as important modulators of ERα transcriptional activity,[9-11] it won't surprise to ascertain that, in ERα+ tumors, FoxO3a could reduce cell migration and invasion through a functional interaction with ERα. On the other hand, in ERα– tumors, the absence of the receptor could enable FoxO3a to trigger some different pathway that leads to an opposite outcome.

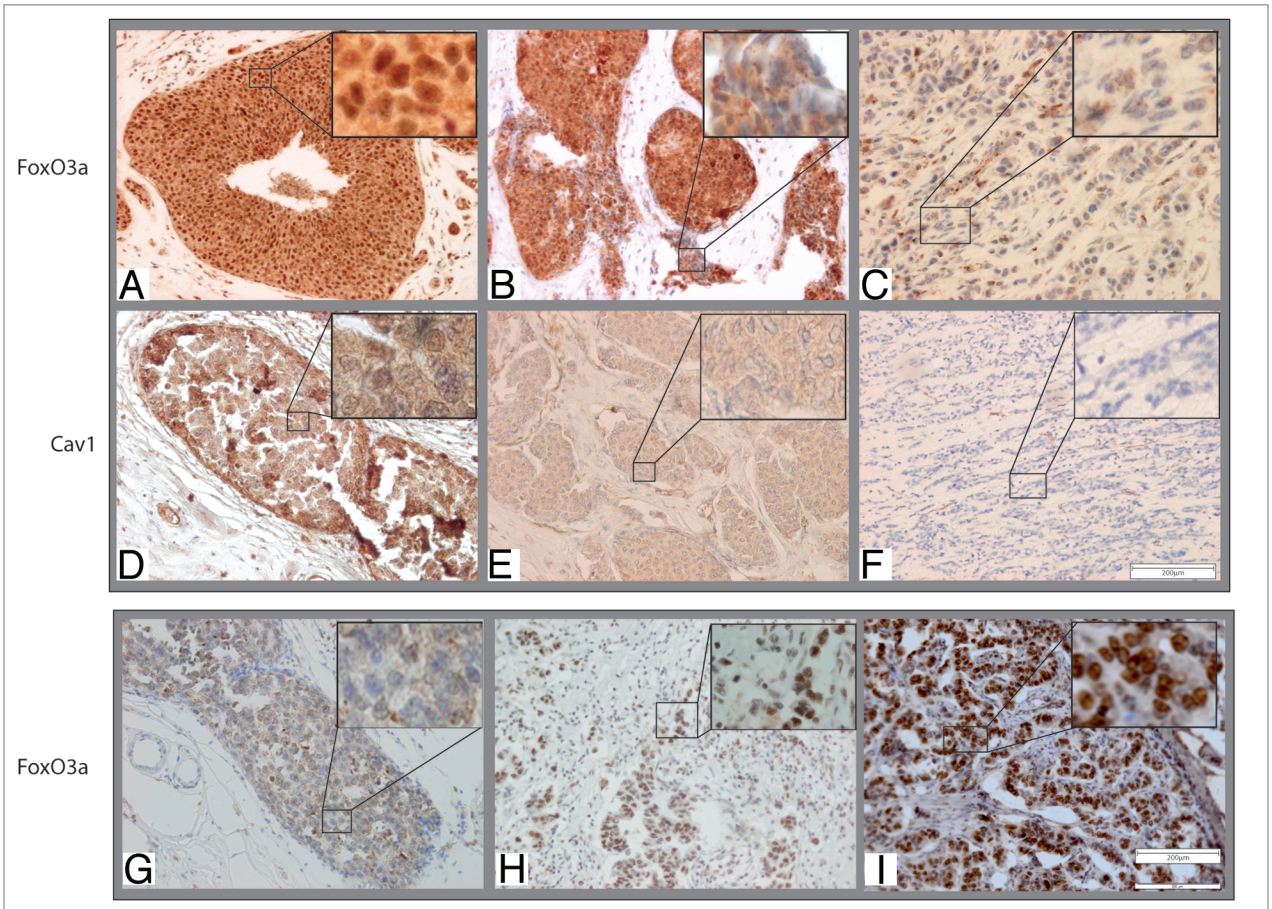
To prove our hypothesis, minimally motile and invasive ERα+ MCF-7 and ZR-75 breast cancer cell lines have been transfected with wild-type F3a and constitutively active F3aAAA mutant, and the effects on cell migration, invasion, and colony formation in soft agar were observed. The results presented here show that FoxO3a overexpression reduces the migratory and invasive potential, as well as anchorage-independent growth (a hallmark of tumor progression), in ERα+ tested cells. It is worth noting that, in all experiments, the constitutively active mutant F3aAAA was always more effective than the wild-type FoxO3a, suggesting that the regulation of the above-mentioned features could occur at the transcriptional level, through the induction of Forkhead-responsive genes. Moreover, the expected reduced motility and invasiveness of ERα+ cells upon E2 stimulation[15] was more evident in F3a and, especially, in F3aAAA-overexpressing cells, providing evidence that E2 and FoxO3a act synergistically on these 2 features (**Fig. 1A and B**; **Fig. S1**, upper panels). On the contrary, E2 stimulation does not show an anti-metastatic behavior in presence of growth factors, since it favors the anchorage-independent growth,[16] suggesting that other growth factors regulated pathways do prevail on that of ERα in the control of this feature. However, in line with our previous observations,[11] FoxO3a overexpression was able to counteract the proliferative effect of E2, and its silencing led to an increase in basal as well as in E2-dependent cell growth (**Fig. 1C$_1$ and C$_2$**). Taken together, these results suggest, once again, that FoxO3a might act as a co-repressor (e.g., by quenching E2/ERα dependent proliferative signals[11]) or a co-activator (e.g., by potentiating E2/ERα mediated inhibition of cell motility and invasion[15]) for ERα.[10]

More importantly, ERα is the key regulator of FoxO3a function, as evidenced by the opposite behavior of overexpressed F3a (and F3aAAA) in ERα-silenced cells if compared with the corresponding ERα-expressing samples (**Fig. 2**). Thus, the lack of the hormone receptor is responsible for the switch of FoxO3a biological function, which shifts from inhibitory (when ERα is present) to stimulatory (when ERα is absent) on cell motility, invasion, and growth in suspension.

This is confirmed by the fact that FoxO3a overexpression exhibits a stimulating (rather than inhibitory as in ERα+ cells) effect on the same features in ERα– MDA-MB-231, MDA-MB-468, and MDA-MB-435S breast cancer cells. Notably, since the results observed in ERα+ and ERα– breast cancer cells following F3a and F3aaAAA ectopic expression, were similar to those obtained in non-breast cancer Ishikawa (ERα+

human endometrial adenocarcinoma) and HeLa (ERα– human cervical cancer) cell lines, respectively, we could assume that FoxO3a controls cell migration, invasion, and growth in suspension with a general, not tissue-specific, mechanism, which seems to depend on ERα expression (**Fig. 3**; **Fig. S1**).

Our results also show how Cav1 represents the ultimate downstream target through which FoxO3a modulates the metastatic potential of ERα+ cells. Cav1 is a multifunctional scaffolding protein that is associated with cell surface caveolae and the regulation of lipid raft domains. Cav1 regulates multiple cancer-associated processes, including cellular transformation, tumor growth, cell migration and metastasis, cell death and survival, multidrug resistance, and angiogenesis. In breast cancer, Cav1 seems to function as a tumor suppressor.[23] In fact, Cav1 mRNA and protein are downregulated or absent in primary human cancers as well as in several mouse and human breast cancer cell lines. Forced re-expression of Cav1 in transformed mammary cell lines abrogates numerous of their tumorigenic properties, including anchorage-independent growth and invasiveness[24] and suppresses growth of breast cancer cell-derived xenografts in nude mice.[25] Moreover, Cav1$^{-/-}$ mice showed an accelerated onset of mammary tumors and lung metastases.[26] In accordance,



J

	Grading				
	G1	G2	G3	Invasive	Total
ER+	5	20	15	25	40
ER-	4	13	23	25	40

K

		FoxO 3a	Cav1
Invasion	r	-0,3535	-0, 4310
	p	0,047	0,0 138
Grading	r	-0, 4123	-0, 5887
	p	0,0 190	0,00 6
Cav1	r	0,6232	
	p	<0,0001	

L

		FoxO 3a
Invasion	r	0, 5805
	p	0,0073
Grading	r	0, 4730
	p	0,0352

Figure 7. Nuclear FoxO3a is highly expressed in non-invasive ERα+, and in invasive ERα– breast tumors. FoxO3a (**A–C**) and Cav1 (**D–F**) expression in ERα+ breast tumors and FoxO3a (**G–I**) in ERα– breast tumor samples. IHC was conducted on tissue sections deriving from biopsies diagnosed as DCIS (**A and D**), microinvasive DCIS (**B and E**), DCIS with contiguous IDC areas (**G and H**) and highly aggressive IDC (**C, F, and I**). Representative fields were photographed at 20× magnification. Insets, showing details of proteins subcellular localization, were taken at 100× magnification. (**J**) Samples descriptions and classification; (**K**) correlation between nuclear FoxO3a or Cav1 content and the tumor grading and invasive potential in ERα+ breast cancer samples; (**L**) correlation between nuclear FoxO3a content and the tumor grading and invasive potential in ERα– breast cancer samples. The correlation coefficient (*r*) and the statistical significance (*P*) are reported.

Cav1 expression has been inversely related to the grade of the primary breast tumors and its upregulation was found to reduce metastasis to distant organs.[21]

In light of this evidence, we questioned if FoxO3a could exert a protective role in ERα+ breast cancer cells through the induction of Cav1 expression. Indeed, in all ERα+ cells tested, FoxO3a overexpression increased the RNA and protein amounts of Cav1, and such increase was additive to that observed under E2 treatment, suggesting that ERα is also involved in the transcriptional induction of Cav1 (**Fig. 4**), which, in turn, seems to be the effector of a less aggressive phenotype, as evidenced by Cav1-silencing experiments (**Fig. 5A–D**) and by the fact that F3a and F3aAAA overexpression failed to inhibit migration, invasion, and growth in suspension in Cav1-negative T47D cells, despite the presence of a low, but still functional, content of ERα (**Fig. 5E–H**).

Since the highest induction of Cav1 has always been observed in F3aAAA-transfected cells, Cav1 regulation by FoxO3a and estrogens at the transcriptional level was investigated. In fact, the 5′-flanking region of the *CAV1* gene, including the promoter region, bear several perfect and predicted forkhead consensus sequences, one of which (at position –1814, located above the promoter sequence) has been reported to be responsible for forkhead dependent *CAV1* gene regulation.[18] However, as the same authors stated, it is possible that other FKHE, also present within the 5′-flanking region, may play a role in Cav1 transcriptional activation by FoxO as well. Indeed, the data presented here clearly show how FoxO3a is able to induce Cav1 transcription by binding to a FKHE motif, mapping nt –305/–299 of its promoter; in addition, the FoxO3a-dependent Pol II recruitment confirms the occurrence of a transcriptional event (**Fig. 6**). To explain the induction exerted by E2, alone or in combination with FoxO3a, on Cav1 expression, we exclude, at the present stage, the direct involvement of ERα in the transcriptional process, since an integrated analysis of ERα binding sites upstream of the Cav1 gene, through Myles Brown lab data sets (http://research.dfci.harvard.edu/brownlab/datasets/index.php?dir=ER_whole_human_genome/)[27] and Cistrome-web application (http://cistrome.dfci.harvard.edu/ap/), evidenced that ERα recruitment to the chromatin occurs at a very large distance from the promoter, on 3 distinct positions around 80–100 Kb upstream of the transcription start site. No ERα binding is reported in the data sets at the promoter level or in its close proximity, as also confirmed by ChIP experiments conducted on several predicted estrogen-responsive motifs identified within the +1/–5000 bp region (data not shown). Additionally, neither Sp1 nor AP-1 transcription factors, 2 well-established mediators of the ERα "non-classical" genomic pathway[28] that have been reported to transcriptionally cooperate with FoxO3a,[29,30] resulted to be involved in Cav1 regulation. In fact, both Sp1 silencing and c-Jun inhibition achieved through the dominant-negative (DN)/c-fos plasmid[31] did not lead to any significant decrease in FoxO3a/E2-dependent Cav1 promoter activation, nor to a reduction of Cav1 protein content (data not shown). Despite these observations, the evidence that liganded ERα induces Cav1 expression, and that E2 and FoxO3a, separately or synergistically, lead to a significant increase of Pol II recruitment on the Cav1 promoter region (**Fig. 6**), suggests that it would be interesting to investigate, by means of the recent and fascinating techniques Chromosome conformation capture (3C) technology and detection of loops in DNA-picked chromatin (DPC),[32,33] if the combined effect of E2 and FoxO3a on Cav1 expression could be ascribed to the interaction of at least one of the 3 above mentioned ERα binding sites, at 80–100 Kb upstream of the transcription start site, where FoxO3a is recruited to the *CAV1* gene promoter (ongoing experiments). In fact, recent studies using tiled microarrays to identify the ERα interacting sites of estrogen responsive genes, showed that EREs can function as enhancer elements far away (up to 100 Kb) from gene promoters, and that other cooperating transcription factors (e.g., FoxA1, AP1 and Sp1) can participate with ERα to regulate the expression of E2-induced genes.[27,34]

Taken together, the results obtained in ERα+ cancer cells show that FoxO3a-dependent decrease of migration, invasion, and colony formation is mediated by both ERα and Cav1, as confirmed by knockout experiments of these two factors (**Figs. 2, 4, and 5**). In particular, ERα cooperates with FoxO3a in the transcriptional induction of Cav1, which, in turn, is responsible of the reduced aggressive phenotype of FoxO3- overexpressing ERα+ cells (**Fig. 8**).

On the other hand, several reports called into question Cav1 role as a tumor suppressor, since it has been found overexpressed in highly aggressive inflammatory breast cancer (IBC) human specimens and cell lines[35] as well as in invasive human breast cancers samples, where its expression was significantly associated with basal-like phenotype, high histological grade, shorter disease-free and overall survival, and, more interestingly, lack of steroid hormone receptors positivity.[36,37] Moreover, in ERα– cancer cells, Cav1 has been found in membrane protrusions, where it promotes tumor cell migration and invasion by regulating either the function of membrane type 1 matrix metalloproteinase (MT1-MMP),[38] or, when phosphorylated (pY14Cav1), the focal adhesion turnover.[22] Therefore, we investigated if the more aggressive phenotype of FoxO3a overexpressing ERα– cells could depend, also in this case, on Cav1 induction. However, no differences in Cav1 levels or phosphorylation status have been detected in ERα– cells following FoxO3a overexpression, nor E2 treatment, possibly through ERβ, has been able to induce Cav1 expression (**Fig. 4**, and data not shown).

Although MMP-9 and MMP-13 induction has been proposed as the mechanism through which FoxO3a increases invasion of cells lacking the hormone receptor,[13] not all the ERα– cell lines tested do express these MMPs, or do express negligible levels. Moreover we failed to detect a reproducible increase in MMP-9 transcripts and in MMP-13 mRNA and protein in FoxO3a-overexpressing cells (data not shown), thus other markers are currently being investigated in our laboratory to justify the higher motility and greater invading ability induced by FoxO3a in ERα– cells. However, it is worth to underline that ERα silencing is a sufficient condition to reverse the effect of FoxO3a on migration, invasion and colony formation in ERα+ cells (**Fig. 2**), thus ERα seems to be a pivotal regulator of FoxO3a function, which switches from protective to malignant depending, respectively,

on the presence or absence of the hormone receptor. A schematic representation of our findings is shown in **Figure 8**.

Finally, an immunohistochemical study from Yoshino's research group showed that nuclear FoxO3a associates with IDC and lymph node metastasis, and the same authors speculated that, in some cases, aberrant activation of FoxO3a may cause the recruitment of metastasis-related molecules, instead of inducing apoptotic genes.[39] Since no association with ERα status has been considered in this study, it might be possible that nuclear FoxO3a could correlate to a more metastatic phenotype only in the subset of ERα– IDC. In line with this hypothesis, nuclear FoxO3a has been recently proposed as a good prognostic factor in luminal-like breast cancer, which contain principally ERα+ cases,[40] where it directly correlates with biomarkers of good prognosis and inversely with mitotic counts and tumor grade. Moreover, with respect to patient outcome, FoxO3a nuclear localization was associated with longer breast cancer specific survival and longer distant metastasis-free interval, independently of the well-established breast cancer prognostic factors.[41]

The screening of nuclear FoxO3a on opportunely selected ERα+ and ERα– tissue samples from patients with breast cancer of ductal origin gave results that perfectly fit with the above-mentioned reports and also confirm the in vitro studies presented in this work. Moreover, the co-expression of Cav1 and FoxO3a in ERα+ tumors, together with the functional link provided by our in vitro data, supports a potentially important role for these 2 proteins in predicting a better tumor prognosis. However, a more systematic evaluation within various subtypes of ERα+ and ERα– non-invasive and invasive breast cancers, in absence or in presence of lymph node and/or long distance metastasis, would help to better clarify the biological and prognostic role of FoxO3a protein expression, also with respect to its subcellular localization. For instance, since no correlation has been found between FoxO3a and ERα [41], the loss of an active (nuclear) FoxO3a might be predictive of a worse phenotype in the subset of ERα+ breast cancers that do not respond to therapy. At the same time, a more accurate immunohistochemical analysis on the biological link between FoxO3a and Cav1 in hormone-positive tumors needs to be addressed. In fact, although Cav1 expression has been associated with lack of the steroid hormone receptor,[37] its positivity in luminal-like tumors could represent a good prognostic factor when associated to a FoxO3a nuclear prevalence.

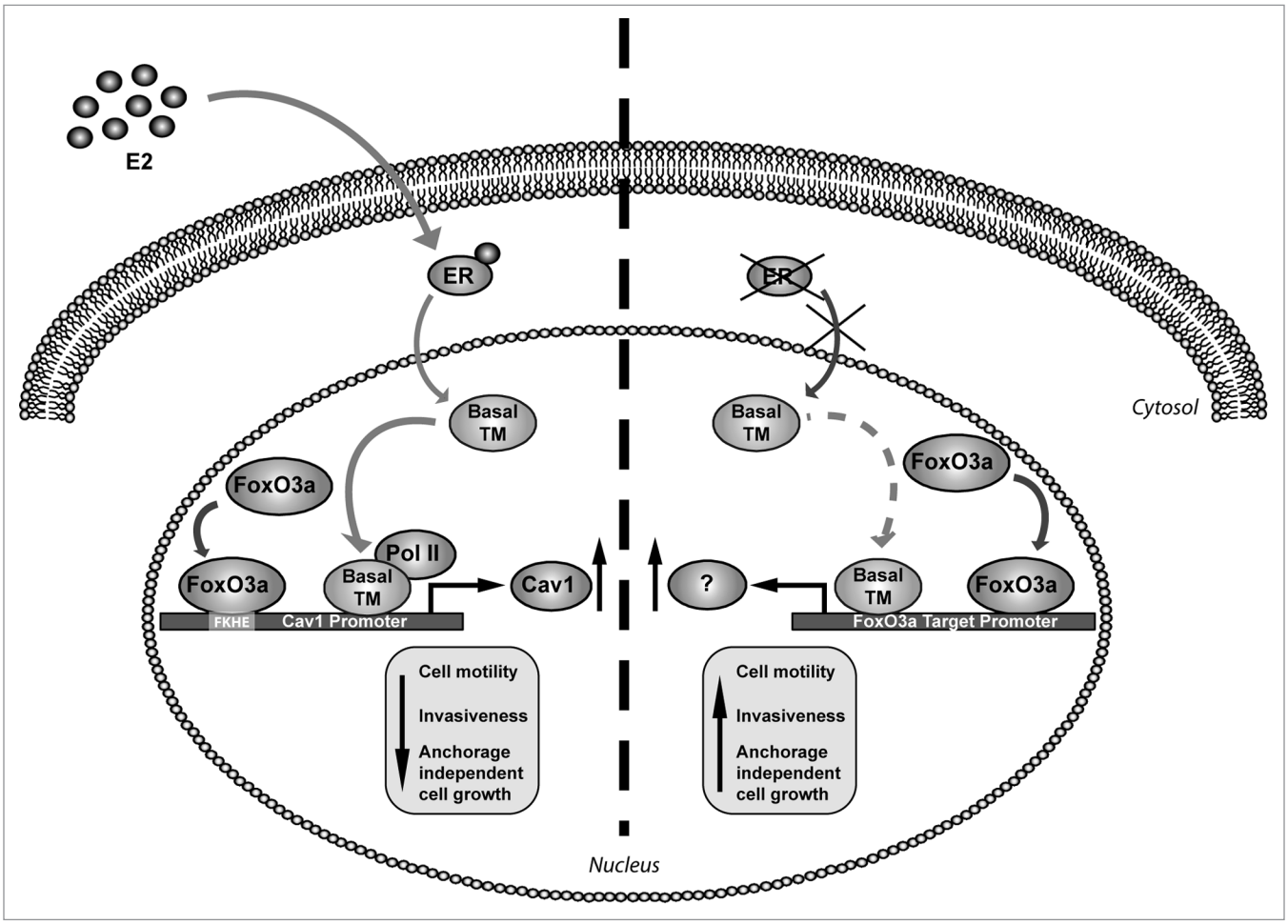


Figure 8. Proposed model for FoxO3a-mediated control of cell motility and invasiveness in presence or absence of ERα. F3a and ERα synergistically induce the expression of Cav1, which, in turn, reduces cell motility and invasiveness of ERα+ breast cancer cells. Transcriptionally active F3a binds to a FKHE located on the Cav1 proximal promoter and increases the recruitment of RNA Polymerase II, which is enhanced upon E2 stimulation. The lack of the hormone receptor enables active F3a to behave in an opposite fashion, thus increasing cell motility and invasion. Basal TM, basal transcriptional machinery.

In conclusion, the results presented here give new insights on the functional role of nuclear FoxO3a, whose overexpression seems to be associated to a low motile phenotype in ERα+ breast cancers and to a more metastatic potential in those lacking the hormone receptor, harboring the idea that ERα may represent the molecular switch determining FoxO3a biological behavior. These evidences clearly suggest that FoxO3a has the potential to become a relevant prognostic factor and a suitable pharmacological target to be exploited in combination therapies for both ERα+ (through FoxO3a activation) and ERα– (through FoxO3a disruption) breast cancer patients.

Materials and Methods

Cell culture, conditions, and treatments

The human breast cancer epithelial cell lines MCF-7, ZR75, T47D, MDA-MB-231, and MDA-MB-468 and the cervical epithelial cell line, HeLa, were purchased from Interlab Cell Line Collection, ICLC, Italy. Ishikawa human endometrial cancer cell line was obtained from D Picard (University of Geneva). MCF-7 and ZR75 were maintained in DMEM/Ham F-12 medium (1:1) (DMEM/F-12) supplemented with 5% FBS. Ishikawa and HeLa cells were grown in MEM containing 10% FBS and 1% non-essential amino acids. MDA-MB-231 and MDA-MB-468 cells were cultured in 10% FBS DMEM. T47D cells were cultured in RPMI containing 10% FBS, 2.5 g/ml glucose, 1% Na-Pyruvate, 10 nM Hepes, and 0.2 U/ml insulin. Additionally, culture media were supplemented with 100 IU/ml penicillin, 100 ng/ml streptomycin, and 0.2 mM L-glutamine. For experimental purposes, cells were synchronized in phenol red-free and serum-free media (PRF-SFM) for 24 h and then, where opportune, switched to PRF-media containing 5% charcoal-treated FBS (PRF-CT) or FBS (ERα+ and ERα– cells, respectively), in presence or absence of 17β-estradiol (E2, Sigma-Aldrich). All media and reagents were purchased from Invitrogen.

Plasmids and transfections assays

The following plasmids were used: pcDNA3 empty vector (Invitrogen); 1038 pcDNA3 flag FKHRL1 (F3a) encoding full-length FoxO3a and 1319 pcDNA3 flag FKHRL1 AAA (F3aAAA), encoding the constitutively active triple mutant of FoxO3a (provided by William Sellers, Addgene plasmids 10708 and 10709,[42] respectively). MCF-7, ZR75, and MDA-MB-231 and MDA-MB-468 cells were resuspended in PRF-growing medium (PRF-GM) and transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, while transfection of T47D, Ishikawa and HeLa cells were conducted with FuGENE HD (Promega). Six hours after transfections, cells were synchronized for 24 h and then subjected either to migration, invasion, and soft agar assays or switched to FBS (ERα– cells) or PRF-CT, in presence or absence of E2 (ERα+ cells), for protein and RNA extraction purposes.

For luciferase assays, the following constructs of the Cav1 promoter[43] were used: pGL3-cavFL, driving the expression of firefly luciferase under the control of the Cav1 promoter full-length (nt –837/–36 from the ATG), pGL3/SRE1/2 (nt –837/–355) and pGL3/SRE3 (nt –354/–36).

Transfections were performed using FuGENE HD. Luciferase activity was measured using the dual-luciferase assay system, normalized to pRL-Tk activity (both from Promega), and expressed as fold-induction over the control.

siRNA-mediated RNA interference

Custom-synthesized siRNA-annealed duplexes (25 bp double-stranded RNA [dsRNA]) were used for effective depletion of FoxO3a (siF3a) and Caveolin-1 (siCav1) transcripts. A scramble siRNA (siScramble) lacking identity with known gene targets was used as a negative control. Cells were transfected in suspension with Lipofectamine 2000 in PRF-GM, using the appropriate amounts of siRNA duplexes (Life Technologies). ERα silencing was conducted according to manufacturer's instructions using siER and the appropriate transfection reagent HiPerFect HTS Reagent purchased from Qiagen. For each silenced gene, at least 2 different siRNAs have been employed with comparable outcome.

Migration and invasion assays

Migration assays were performed as previously described.[15] Briefly, 6 h after transfection or silencing, cells were serum starved for 24 h, resuspended in PRF-SFM, and seeded (10^4 cells/insert) on the upper face of 24-well modified Boyden chambers (8 μm) (Corning); 500 μl of 5% PRF-CT with or without 100 nM E2 (for ERα+ cells) or PRF-GM (for ERα– cells) were added to the bottom of the wells. After opportune incubation, migrated cells were stained with Coomassie brilliant blue and counted under the microscope.

For invasion experiments, 30 μl of Matrigel™ Basement Membrane Matrix (BD Biosciences) (1:3 dilution in PRF-SFM) were coated on the internal surfaces of the Boyden chambers and let solidify at RT for 30 min. The lower chambers were loaded as described for migration assays. Cells suspended in 200 μl of 1% PRF-CT (ERα+ cells) or 1% FBS (ERα– cells), respectively, were plated into the upper chambers (10^5 cells/insert). After the appropriate times of incubation, cells in the upper chamber were removed by a cotton tip; membranes were then mixed in methanol for 10 min at –20 °C, rinsed with PBS, stained with DAPI (Sigma Aldrich, Italy) for 5 min, rinsed again in PBS and dried. The filters were then detached from the chamber, and mounted onto slides using Fluoromount mounting medium (Sigma Aldrich) and observed under a fluorescence microscope (Olympus BX51 fluorescence microscope, Olympus Italia srl). Invading cells were photographed at 10× magnification using ViewFinder™ Software, through an Olympus camera system dp50 and then counted using ImageJ software (NIH).

Anchorage-independent growth assay

Transfected or silenced ERα+ cells were seeded in 1 mL of 0.3% GellyPhor™ HR agarose (Euroclone S.p.A.) on top a base of 0.6% agarose in 12-multiwell plates in PRF-CT (2×10^4 cells/well) and treated with 100nM E2 or left untreated; ERα– cells were seeded in PRF-GM (3×10^4 cells/well). On day 14, the colonies (>50 μm) were exposed to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h, photographed at 4× magnification and counted under the microscope (Olympus BX51 microscope).

RNA extraction, reverse transcription, and real-time (RT)-PCR

Total RNA was isolated using TRI-reagent (Ambion) and treated with DNase I (Life Technologies). Two micrograms of total RNA were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was diluted 1:3 in nuclease-free water, and 5 μl were analyzed in triplicate by RT-PCR in a iCycler iQ Detection System (Bio-Rad) using SYBR green Universal PCR Master Mix (Bio-Rad) and the following pairs of primers: FoxO3a forward 5′- CAAACCCAGG GCGCTCTT-3′ and reverse 5′- CTCACTCAAG CCCATGTTGC T-3′ (68 bp); Cav1 forward 5′- CAGTTTTCAT CCAGCCACGG-3′ and reverse 5′- CGGATGGGAA CGGTGTAGAG-3′ (82 bp).

Negative controls contained water instead of first-strand cDNA. Each sample was normalized on its 18S rRNA content. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. The final results were expressed as *n*-fold differences in gene expression relative to 18S rRNA and the calibrator, calculated using the $\Delta\Delta C_T$ method as follows: *n*-fold = $2^{-(\Delta C_T \text{ sample} - \Delta C_T \text{ calibrator})}$, where the ΔC_T values of the sample and calibrator were determined by subtracting the average C_T value of the 18S rRNA reference gene from the average C_T value of the different genes analyzed.

Western blotting (WB) assays

Protein expression was assessed by WB assay as previously described.[44] Total lysates were extracted using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate) plus inhibitors (0.1 mmol/liter Na_3VO_4, 1% PMSF, and 20 mg/ml aprotinin). The protein content was determined using Bradford dye reagent (Bio-Rad). Fifty μg of lysates were separated on an 11% polyacrylamide denaturing gel and transferred to nitrocellulose membranes. Proteins of interest were detected with specific polyclonal (p) or monoclonal (m) antibodies (Abs), recognized by peroxidase-coupled secondary Abs, and developed using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech). The following Abs were used: anti-FoxO3a (75D8) pAb (Cell Signaling), anti-Cav1 (N-20) pAb, anti-ERα (F-10) mAb, and anti-GAPDH (FL-335) pAb (Santa Cruz Biotechnology). Images were acquired by using an Epson Perfection scanner (Epson).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as previously described.[11] The immuno-cleared chromatin was precipitated with anti-FoxO3a pAb (Abcam, USA) and anti-Polymerase II (N-20) pAb (Santa Cruz Biotechnology). Normal rabbit IgG (Santa Cruz Biotechnology) was used instead of primary Abs as negative controls. Immunoprecipitated DNA was analyzed by RT-PCR, as described above. A pair of primers (5′-GAGATGATGC ACTGCGAAAA-3′ and reverse 5′-GCCAAAGGTT TGTTCTGCTC -3′) (242 bp) mapping the FKHE-containing Cav1 promoter region forward was used.

Tissue collection, immunohistochemistry (IHC), and data analysis

Formalin-fixed paraffin-embedded tissue sections were prepared from primary operable breast cancer cases (15 DCIS and 25 IDC from ERα+ tumors and an equal number from ERα– tumors) from patients under age 80 who underwent mastectomy at the Cosenza Hospital (Cosenza Hospital Authority) between 2011 and 2012. FoxO3a, ERα and Cav1 expression were assessed by IHC. The rabbit anti-FoxO3a pAb (cat. PA1-14171, Thermo Scientific) and the rabbit anti-Caveolin-1 pAb (N-20) (sc-894, Santa Cruz Biotechnology) were optimized at a working dilution of 1:200 in Dako Real antibody diluent (DAKO); the mouse anti-ERα (Clone 1D5, DAKO) was ready to use. Deparaffinization, rehydration, and antigen unmasking was obtained by incubation in tris-phospahte buffer (Envision Flex target retrieval solution) in a Pre-Treatment Module for Tissue Specimens (PTLINK), according to the manufacturer's instructions (DAKO). The staining was performed in a Dako Autostainer Link48 immunostainer, using a linked streptavidin biotin technique (Envision Flex kit High pH, DAKO) in accordance with the manufacturer's instructions. Sections were counterstained in hematoxylin and coverslipped using DPX mounting medium (both from Sigma-Aldrich).

The expression and subcellular localization of FoxO3a and Cav1 were evaluated microscopically. Pictures of representative fields were taken at opportune magnification using ViewFinder™ Software, through an Olympus camera system dp50.

Ethical statement

The clinical investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki of 1975 and to national and international guidelines and has been approved by the Research Ethics Committee of Cosenza Hospital Authority. The informed consent was not requested, since the study was retrospective and the data were analyzed anonymously.

Statistical analysis

All data were expressed as the mean ± s.d. of at least 3 independent experiments. Statistical significances were evaluated using Student *t* test. The correlations between nuclear and cytoplasmic FoxO3a and Cav1 with respect to tumor grading and invasiveness were examined with Pearson correlation test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26421

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