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Leptin Mediates Hyperglycemia-induced Angiogenic Effects in Retinal Endothelial Cells

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ABSTRACT

Hyperglycemia (HG)-activated cytokines and inflammatory factors have been implicated in ocular neovascularization and diabetic retinopathy (DR). However, the effects of HG on the expression and function of leptin in retinal cells have never been investigated. We found that in RF/6A retinal endothelial cells, chronic high glucose (30 mM/L) exposure significantly increased leptin mRNA expression and upregulated leptin protein and leptin receptor levels. Furthermore, HG potentiated RF/6A cell migration, chemokinesis, and angiogenic differentiation. These effects coincided with the activation of several intracellular pathways implicated in angiogenic and metabolic response, i.e., STAT3, ERK1/2, Akt, and AMPK, increased levels of glucose response protein and COX2 as well as modulation of the expression of PAI-2 and HIF-1α. All pro-angiogenic processes promoted by HG and several of HG-activated intracellular pathways were partially or totally blocked in the presence of the leptin receptor antagonist peptide. The results demonstrate for the first time that the leptin/leptin receptor axis is implicated in HG-induced biological effects in retinal endothelial cells. Thus, targeting leptin pathways might represent a novel avenue in the treatment of ocular neovascularization.

INTRODUCTION

❖ Diabetic retinopathy: Epidemiology

DR is one of the most common microvascular complications of diabetes, affecting almost 100% of patients with type 1 diabetes (T1DM) and more than 60% of those with type 2 diabetes (T2DM) [1–4].

In many countries, DR is the most frequent cause of preventable blindness in working-aged adults between 20 and 74 years [5]. According to World Health Organization Report [6] in January 2011, over 220 million people worldwide had diabetes and the prevalence will rise to 366 million by 2030 [7,8]. For example, in USA diabetes affects 6.3% of the total population [1]. Among this population an estimated 40% (8% for vision-threatening retinopathy) of people with T2DM and 86% (42%) with T1DM have DR [9,10]. In Italy, out of 6.857 patients screened in 1992–2003, the prevalence of DR was 39%, of which 19% was classified as mild, 11% as moderate, and the rest as more severe [8].

Many studies have provided evidence that DR is not only a significant cause of acquired visual loss and blindness but it is also linked to three-fold excess risk of coronary disease, ischemic stroke [11–14] and heart failure, independent of cardiovascular risk factors [15,16]. These findings suggest that the presence of DR is a sign of widespread end-organ microcirculatory damage in people with diabetes, and that there is the need for improvement in careful cardiovascular monitoring and follow-up for patients with DR [16]. In addition epidemiological studies have also shown that DR is associated with many other systemic and lifestyle factors, including nephropathy [17], obesity [18], alcohol consumption [19], and hematological markers of anemia [20], hypothyroidism [21], inflammation and endothelial dysfunction [22]. However, some of these findings have been inconsistent, and the precise role of factors related to these diseases in the pathogenesis of DR is not well defined [4].

❖ **Pathogenesis of diabetic retinopathy**

Understanding the etiology and pathology of DR is critical to the development of modern treatments of this condition. For instance, one of the major factors believed to play a role DR is chronic exposure to HG and other causal risk factors (eg. hypertension) is believed to initiate a cascade of biochemical and physiological changes that ultimately lead to microvascular damage and retinal dysfunction [4]. In fact, clinical studies have demonstrated a strong association between DR and glycemic exposure [23], glycated hemoglobin (HbA1c) levels and duration of diabetes [24,25]. For instance, every percent reduction in HbA1c (eg, from 9% to 8%) lowers risk of DR by 30–40% and the effect appears to be long-lasting (metabolic memory) [26]. A recent meta-analysis [27] of three large population-based studies of DR showed a graded relation between the level of glycemia and frequency of DR signs, even below the diagnostic criterion for diabetes (fasting plasma glucose of 7·0 mmol/L). These findings suggest that further reduction in glycemic levels might have additional benefits for DR in people with diabetes [4]. In support of these findings, improvements in glycemic control have been correlated with a reduced burden of DR in T2DM [28], and with a decreased risk of retinal photocoagulation [29].

In addition to HG, hypertension has been implicated in DR through increased blood flow and mechanical damage (stretching) of vascular endothelial cells, enhanced release of vascular endothelial growth factor (VEGF) [25,30]. Every 10 mm Hg increase in systolic blood pressure is associated with roughly 10% excess risk of early DR and a 15% excess risk of proliferative retinopathy (PDR) [25,31]. In the United Kingdom Prospective Diabetes Study (UKPDS), tight blood pressure control reduced the risks of DR progression by about a third, visual loss by half, and the need for laser treatment by a third in people with T2DM [5]. However, these benefits were not sustainable without continuing and long-term maintenance of blood pressure control [32]. Some blood-pressure-lowering drugs, such as rennin-angiotensin inhibitors, could have benefits beyond their blood-pressure-lowering effects [4].

Structural and functional changes in the retinal vasculature are closely related to diabetes and DR [33]. Recent advances in computer-based retinal image analysis have allowed quantitative assessment of the retinal vasculature to study these changes in greater detail. For example, widened retinal arteriolar caliber has been associated with the development of DR in both T1DM and T2DM [34–36]. Retinal arteriolar dilatation might be an early physiological indicator of microvascular dysfunction [37], signifying impaired arteriolar autoregulation. Retinal arteriolar dilatation has been further postulated [36], according to the laws of Starling and Laplace, to increase retinal capillary pressure, leading to capillary wall dilatation (micro aneurysms), leakage (edema and hard exudates), and rupture (hemorrhages) [4]. Another factor implicated in DR is dyslipidemia [5]. For example, in the Diabetes Control and Complications Trial (DCCT), researchers showed that severity of DR was associated with increasing triglycerides and inversely associated with HDL cholesterol [38]. Thus the optimum control of blood glucose, blood pressure, and possibly blood lipids remains the foundation for reduction of risk of DR development and progression [4].

- **Molecular mechanisms implicated in retinopathy**

Endothelial cell injury and pathological angiogenesis represent central events in the development of DR and other eye-related complications [39]. However, the molecular mechanisms by which HG may induce ophthalmic disease are not fully understood. Experimental evidence suggests that HG can induce several processes involved in ocular neoangiogenesis, such as endothelial cell differentiation, migration, and may impact vascular and retinal permeability [40–42]. However, the impact of HG on endothelial cell proliferation is debatable [42–44]. HG effects in endothelial cells have been associated with the activation of different intracellular signals, such as the extracellular signal regulated kinase (ERK1/2), phosphoinositide 3-kinase/AKT kinase (PI3K/AKT), and signal transducer and activator of transcription 3 (STAT3) pathways implicated in mitogenic, motogenic, and angiogenic signaling [43, 45–48]. In addition, in different cell types, HG has been shown to

modulate the expression or activity of transcription factors such as hypoxia-inducible factor 1α (HIF-1 α) [49–52] and cAMP responsive element binding protein (CREB) [51], several mediators of inflammation such as cyclooxygenase-2 (COX2) [43], and endoplasmic reticulum (ER) stress and immune response effectors (glucose-regulated protein 78, GRP78 and RNA-dependent protein kinase-like endoplasmic reticulum kinase, pPERK [45]. Furthermore, hyperglycemic conditions may trigger the activation of non-canonical endoplasmic reticulum stress mediators, such as the multifunctional protein PAI-2 (plasminogen activator inhibitor type 2) [53].

- **VEGF and anti-VEGF therapies and their limitation**

One of the recognized mechanisms by which HG might induce DR is increased expression of various cytokines and inflammatory factors, most notably VEGF [40,54,55]. VEGF is a potent, diffusible, endothelial-specific mitogen that is released in response to hypoxia and upon binding to the VEGF receptor 2 (VEGFR-2), expressed by the vascular endothelium, elicits angiogenesis and vascular hyperpermeability [54]. The pathologic transformations of the retinal vasculature seen in intraocular vascular disease are associated with increased expression of VEGF-A. In model systems, VEGF alone is sufficient to trigger intraocular neovascularization, and its inhibition is associated with functional and anatomic improvements in the affected eye. Therapeutic interventions targeting VEGF include intraocular capture and neutralization by engineered antibodies or chimeric receptors, downregulation of its expression with steroids, or alleviation of retinal ischemia, a major stimulus for VEGF expression, with retinal ablation by laser treatment. Data from prospective randomized clinical trials indicate that VEGF inhibition is a potent therapeutic strategy for intraocular vascular disease. These findings are changing clinical practice and are stimuli for further study of the basic mechanisms controlling intraocular angiogenesis [54].

However, several other activators of angiogenesis such as platelet-derived growth factor, basic fibroblast growth factor (bFGF), hepatocyte growth factor, interleukins 1a, 6 and 8, and leptin

have also been implicated in the development of DR [56,57]. Many of these factors act through upregulation of VEGF synthesis but their direct involvement remains largely unclear [56–58]. At present, VEGF targeting drugs, ranibizumab (a humanized monoclonal antibody fragment with molecular weight of 48 kDa), and bevacizumab (a recombinant humanized monoclonal antibody with molecular weight of 149 kDa), that were engineered to bind with high affinity and to neutralize all biologically active isoforms of VEGF [57,59] and aflibercept (a fusion protein containing the second immunoglobulin domain of VEGFR-1 and the third immunoglobulin domain of VEGFR-2, which binds all isoforms of VEGF, VEGF-B, and placental growth factor) [54,57,60] are approved for the treatment of wet age-related macular degeneration (AMD) and diabetic macular edema (DME), and experimentally used for other eye diseases, e.g. PDR [57,61]. Most trials have shown some benefits with the use of intravitreal anti-VEGF agents for both DME and PDR [4,57].

However, adverse effects (systemic and ocular) and development of resistance to the treatment have been noted with long-term use [56,62,63]. Local adverse events of intravitreal anti-VEGF therapy determine cataract formation, retinal detachment, vitreous hemorrhage, infection, and potential loss of neural retinal cells [64]. Furthermore, a significant portion of anti-VEGF agents injected into the eye could pass into the systemic circulation [64,65]. Thus, systemic inhibition of angiogenesis is a potential risk, which could compromise critical vascular responses to ischemic events in patients with diabetes. Other unwanted systemic side-effects can be hypertension, proteinuria, and impaired wound healing [64], which are also of relevant concern for patients with diabetes. Although clinical trials on the use of intravitreal anti-VEGF therapy for treatment of AMD generally show low (0·6–1·2%) rates of stroke [66], this risk could be increased in patients with DR because of pre-existing diabetes-related vascular disease [56]. Thus, both clinicians and patients should recognize and weigh the risks and benefits of these agents when they are used to treat DR.

In this context, targeting proangiogenic factors other than VEGF could be prove to be an effective alternative or complementary therapy for pathological neovascularization in the eye. As

research continues to broaden our pathogenic understanding of DR, new treatment modalities are expected to emerge [4].

- **Leptin and its angiogenic activity**

Notably, several studies suggested a role of leptin, an angiogenic and pro-inflammatory cytokine in ocular neovascularization, including DR [56,67–71]. A recent study has revealed that plasma leptin concentrations are elevated significantly in patients with PDR relative to those with non proliferative retinopathy (NPDR) [70, 72]. However, whether leptin is causally related to the progression of DR is currently undefined [70]. Leptin stimulates the ischemia-induced retinal neovascularization possibly through the upregulation of VEGF in retinal endothelial cells, thereby suggesting that leptin antagonism may offer a novel therapeutic strategy to treat DR [70].

While exposure to HG has recently been shown to modulate leptin and its receptor (ObR) in non-ocular models, i.e., epidermal cancer cells [73], cardiomyoblasts [74], and human GnRH-secreting neurons [75], the effects of HG on leptin/ObR expression in ocular tissues or ophthalmic cell models have never been addressed.

We have recently demonstrated that leptin is a potent angiogenic factor in retinal and corneal endothelial cells and that ObR antagonist inhibits leptin-induced effects in these models [57]. However, the potential effects of HG on the expression and function of leptin in retinal cells has never been investigated. Consequently, this study evaluated the effects of chronic high glucose exposure, on migration, chemotaxis and chemokinesis, tube formation, as well as leptin mRNA and protein expression, in retinal endothelial cells addressed the potential of the peptide-based ObR antagonist, Allo-aca, to inhibit these effects.

Leptin, a pluripotent cytokine has been first described as an adipocyte-derived hormone that regulates energy expenditure and food intake via hypothalamic effects [57,76,77]. Later studies proved that leptin is expressed by different peripheral organs and tissues and is involved in many

physiological and pathological processes, most notably: immune response, hematopoiesis, fertility, bone remodeling, cardiovascular disease, T2DM, and cancer [57,78,79]. Of special interest is the ability of leptin to regulate normal and abnormal angiogenesis [57].

The Ob-R is a single transmembrane protein that belongs to the gp130 family of cytokine receptor superfamily [70]. The ObR has several alternatively spliced isoforms, one of which, a biologically active Ob-Rb isoform, is expressed not only in the hypothalamus but also in a variety of peripheral tissues, suggesting the direct action of leptin in the periphery. Studies in vitro demonstrated that the peripheral actions of leptin include the activation of platelet aggregation [80], the modulation of immune function [81], and the stimulation of VEGF and angiogenesis [82,83]. Most of these studies were carried out using human umbilical vein endothelial cells (HUVEC) or aortic endothelial cells [84,85] only one study involved retinal endothelial cells [70]. In HUVEC, the use of specific inhibitors suggested that leptin-mediated angiogenesis depends on ObR crosstalk with VEGFR2. Interestingly, some studies show that leptin-induced angiogenesis in HUVEC can be partially reduced with VEGFR inhibitor [86], while others did not observe such effects [87], suggesting independent leptin action [57].

- **Anti-leptin therapy as potential diabetic retinopathy treatment**

We recently generated peptide-based compounds that interfere with leptin/ObR binding and downstream signaling [88,89]. The lead ObR antagonist Allo-aca is a 9 residue peptidomimetic that binds ObR in vitro, inhibits leptin-induced proliferation and signaling at pM-low nM concentrations in vitro and reduces the growth of ObR-positive tumor xenografts in vivo at 0.1-0.5 mg/kg/day doses [85,90,91]. Allo-aca was also used to assess the importance of leptin in the growth, signaling and angiogenesis of retinal and corneal cells in vitro [57].

❖ **Summary**

DR is a common microvascular complication that is not only a serious threat to vision, but may also signify an increased risk of morbidity and mortality attributable to systemic vascular complications. DR may, therefore, represent vascular damage and injury not only in the eyes but also in other vital organs such as the brain, heart, and kidneys in people with diabetes. For ophthalmologists, physicians, and other healthcare providers, it is important not to overlook the broader associations and clinical implications of DR [4].

Timely laser therapy is effective for preservation of sight in PDR and DME, but its ability to reverse visual loss is poor. Vitrectomy surgery might occasionally be needed for advanced retinopathy. New therapies, such as intraocular injection of steroids and anti-VEGF agents, are less destructive to the retina than are older therapies, and could be useful in patients who respond poorly to conventional therapy. The outlook for future treatment modalities, such as inhibition of other angiogenic factors, regenerative therapy, and topical therapy, is promising [4].

MATERIAL AND METHODS

❖ Reagents

The ObR antagonist, Allo-aca, is a short leptin-based peptidomimetic (H-alloThr-Glu-Nva-Val-Ala- Leu-Ser-Arg-Aca-NH_2) whose sequence is based on leptin/ObR binding site III. The design and efficacy of Allo-aca in vitro and in vivo have been reported by us before [57,90,92,93]. Leptin (human recombinant) was purchased from R&D Systems (Minneapolis, MN).

❖ Cell lines and growth conditions

Monkey endothelial retinal cells (RF/6A) were purchased from the American Type Culture Collection (Rockville, MD, USA). RF/6A cells were grown in Minimum Essential Medium (MEM) containing 1 g/L glucose, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). In hyperglycemia experiments, the cells were cultured in serum-free medium (SFM: MEM with 10 µM $FeSO_4$, 0.5% bovine serum albumin, 1% FBS, 1% P/S) containing 30 mM D-glucose (G30). SFM containing 30 mM mannitol was used as osmotic control. All cell culture reagents were purchased from Cellgro (Herndon, VA, USA).

❖ Proliferation assay

The cells (2-5th passage) were plated in 24 well plates at concentrations 8 x10^4 cells/well. At 70% confluence, the cells were shifted to SFM for 24 h and then cultured for 24, 48, 72 h either in SFM or G30 in the presence or absence of 10-250 nM Allo-aca. Cell numbers before and after treatments were determined by direct counting with trypan blue exclusion. All assays were done in triplicate and repeated 3-6 times. The percentage decrease/increase in cell number vs. control SFM was calculated and expressed as mean ± standard deviation (SD).

❖ **Wound-healing assay**

Directional cell migration in vitro was assessed using a wound-healing assay. Linear scratches (3 per plate) were produced in 100% confluent cultures of RF/6A cells using a 200 µl tip. The cultures were then incubated for 24 h either in G30 with or without 10-250 nM Allo-aca, SFM containing 250 ng/ml leptin with or without 10-250 nM All-aca, or SFM with 30 mM mannitol. After 24 h, wound dimensions (at least 6 fields/experimental condition) were photographed using Olympus 1x81 phase-contrast microscope at 2.0X magnification and images were acquired using Metamorph 7.5 program. The scratch areas were quantified using Adobe Acrobat Pro program.

❖ **Transwell migration assays**

The effects of hyperglycemia and leptin and leptin on chemotactic and chemokinetic properties of RF/6A cells were studied using Transwell inserts (8.0µm pore size) (Corning, Tewksbury, MA). The cells were plated in at 5-8x10^4 cells/well and allowed to migrate through the membrane for 24 h. Then, non-migrated cells in the upper chamber were removed and the cells that migrated across the membranes were stained with Giemsa for 20 min and counted. To test chemokinetic effects of leptin or hyperglycemia on RF/6A cells, 50-250 ng/mL leptin or G30 were added in both chambers. To test chemotactic effects of leptin on RF/6A cells, increasing leptin concentration (50, 100, 250 ng/ml) were added to the lower chamber only. The involvement of ObR in leptin- or G30-dependent chemotaxis or chemokinesis was assessed using 50-250 nM Allo-aca. Each migration assay was done in triplicate and repeated at least 3 times and the mean number of migrated cells ± SD was determined.

❖ **Angiogenic assay**

The ability of cells to organize into enclosed spaces (ES) was carried out as described in detail previously [57]. The cells were first exposed to SFM, G30, or G30 plus 250 nM Allo-aca for 24

h. Next, pretreated cells ($1x10^4$) were suspended in 200 µl of either SFM, G30 or G30 plus 250 nM Allo-aca, seeded in 96 well plates covered with polymerized growth factor-reduced Matrigel matrix (BD Biosciences, Franklin Lakes, NJ) and incubated for 3 h at 37°C to allow formation of ES. The process was recorded with Olympus 1x81 phase-contrast microscope at 3.2X magnification using the Metamorph 7.5 program. The number of ES in the whole photographed area (central 70% of the well) was scored by two observers. Each experiment was done in triplicate and repeated at least 3 times. The mean number of ES ± SD was determined for each condition.

- **Quantitative Real Time PCR (qRT-PCR)**

RF/6A at 70% confluence were shifted to SFM for 24 h and then treated with either G30 or G30 plus 250 nM Allo-aca for 24 and 72 h. RNA was isolated using Trizol Reagent (Life Technologies, Grand Island, NY) according to manufacturer's instructions. A total of 4 µg of RNA was reverse transcribed in 20 µL of reaction volume using the High-Capacity cDNA Kit (Life Technologies). Four µL of the RT product were used to amplify leptin sequences using TaqMan probes Rh02788316_m1 for monkey leptin (Life Technologies). To normalize qRT-PCR results, parallel reactions were run on each sample for β-actin using a TaqMan probe (Life Technologies). The levels of target mRNA relative to β-actin mRNA were determined using a comparative CT method, as suggested by the manufacturer (Life Technologies). All reactions were done in triplicate and an average CT value (±SD) for all RNAs was calculated. The individual experiments were repeated at least 3 times.

- **Immunofluorescence**

Leptin protein was detected in RF/6A cells by immunofluorescence (IF), as described by us before [57,94,95]. In short, $1x10^5$ cells were plated on glass coverslips in normal growth medium. After 24 h, the cells were synchronized in SFM for 24 h and then treated either with G30 or 250 ng/mL

leptin in the absence or presence of 250 nM Allo-aca for 72 h. Next, the cells were washed with PBS, fixed in methanol at -20°C for 10 min, and permeabilized in 0.2 Triton X-100% for 5 min at room temperature. Leptin expression was detected using pAb A-20 (1:25 dilution; 2 h) and goat anti-rabbit IgG-FITC (1:1000 plus 1.5% blocking goat serum; 1 h). In control experiments, primary Abs were replaced by non-immune serum. Following staining, the coverslips were mounted using UltraCruz Mounting Medium containing 1.5 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) to allow visualization of cell nuclei. The expression of leptin was assessed using Olympus 1x81 phase-contrast microscope at 3.2X magnification. The percentage of positive cells was determined in 10 visual fields. All Abs and other reagents were purchased from Santa Cruz Biotechnology (Dallas, TX).

- **Intracellular signaling**

To assess the effects of high glucose on RF/6A cells, 70% confluent cell cultures at 4th or 5th passage were shifted to SFM for 24 h and then treated with G30 in the presence or absence of 250 nM Allo-aca for 24, 72 or 96 h, or were left untreated. Next, the cells were lysed and total cellular proteins were obtained as described previously [57]. The expression of ObR and downstream signaling molecules was evaluated by Western Blot (WB) in 50-100 μg of total proteins. The following primary antibodies (Abs) from Cell Signaling Technology (Danvers, MA) were used: phospho-Akt, Akt Ser473 pAb, 1:500; total Akt, Akt pAb, 1:1000; phospho-STAT3, STAT3 Tyr705, D3A7 mAb, 1:500; total STAT3, STAT3 79D7 mAb, 1:500; phospho-ERK1/2, p44/42 mitogen-activated protein kinase (MAPK; ERK1/2) pAb Thr202/Tyr204, 1:1000; total ERK1/2, p44/42 MAPK pAb, 1:1000; total COX2, COX2 pAb, 1:250, total AMPK-α, 1:500**.** The following primary Abs from Santa Cruz were used: ObR, H-300 pAb, 1:500; HIF-1α, H-206, 1:500, phospho-AMPKα 1/2, 1:500; CREB-2 C-20, 1:500; GRP 78 A-10, 1:1000; PAI-2 N-18, 1:400; phospho-

PERK Thr 981, 1:500; PERK H-300, 1:1000. The intensity of bands corresponding to studied proteins was measured using Image J program as described before [57].

RESULTS

- **Hyperglycemia stimulates leptin expression in RF/6A retinal endothelial cells. ObR antagonist, Allo-aca inhibits this effect.**

First, we assessed if HG is able to affect leptin expression in RF/6A retinal endothelial cells. The exposure of the cells to G30 increased leptin mRNA levels at 24-72 h (**Figure 1**). The greatest increase (~3-fold) was observed at 72 h of treatment, while at earlier time points, G30 induced leptin expression by ~50%. The effects of G30 at 24 and 72 h were statistically significant vs. SFM ($p<0.05$) (**Figure 1**). The increased leptin mRNA expression in RF/6A cells under HG conditions was paralleled by elevated cytoplasmic leptin protein content. At 72 h of G30 treatment, ~30% of cells displayed strong cytoplasmic leptin staining as assessed by IF, while leptin was noticeable only in ~5% of untreated cells (**Figure 2**).

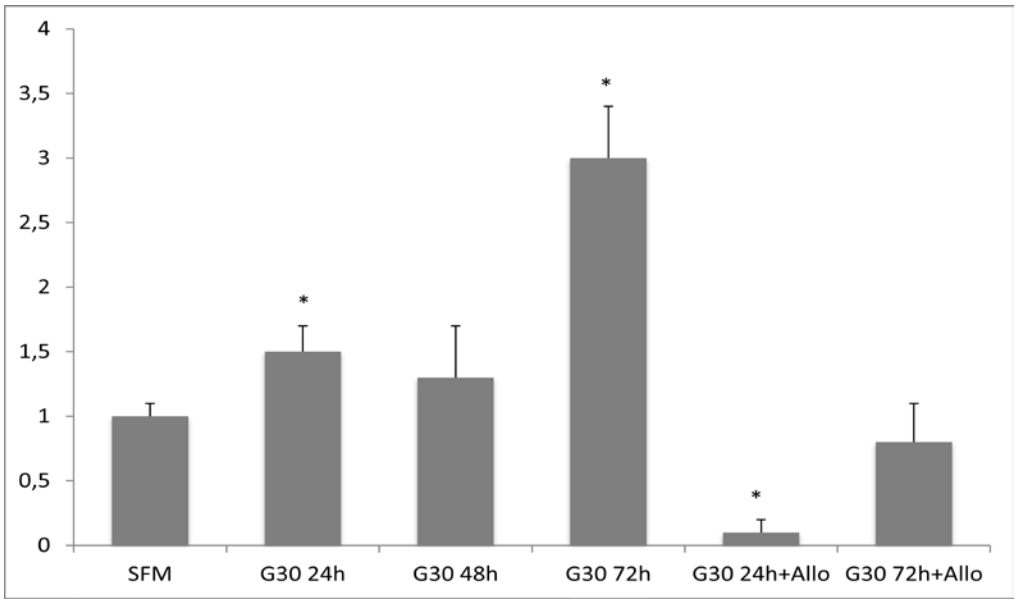

Figure 1. Effects of high glucose and Allo-aca on leptin mRNA expression in RF/6A cells. RF/6A cells were treated with high glucose medium (G30) or G30 plus 250 nM Allo-aca (G30+Allo-aca) for 24-72 h. The expression of leptin mRNA was assessed by QRT-PCR as described in Materials and Methods. The values represent fold increase (± SD) of leptin mRNA levels in HG treated cells vs. untreated controls (SFM) assigned value 1. Statistically significant changes ($p\leq0.05$) vs. SFM are marked with asterisk.

We reported previously that leptin can induce its own expression in retinal endothelial cells, leading to the activation of the autocrine leptin/ObR axis [57]. Here we investigated if the effects of G30 on leptin expression could be inhibited by the ObR antagonist, Allo-aca. We found that in RF/6A cells, Allo-aca at 250 nM concentration totally blocked G30-mediated leptin mRNA expression at 72 h and decreased leptin mRNA to below basal levels at 24 h (**Figure 1**). Furthermore, 250 nM Allo-aca downregulated G30-induced leptin protein expression (**Figure 2**). Similar effects on G30-dependent leptin expression were achieved with Allo-aca at 100 nM, but not at lower concentrations (data not shown).

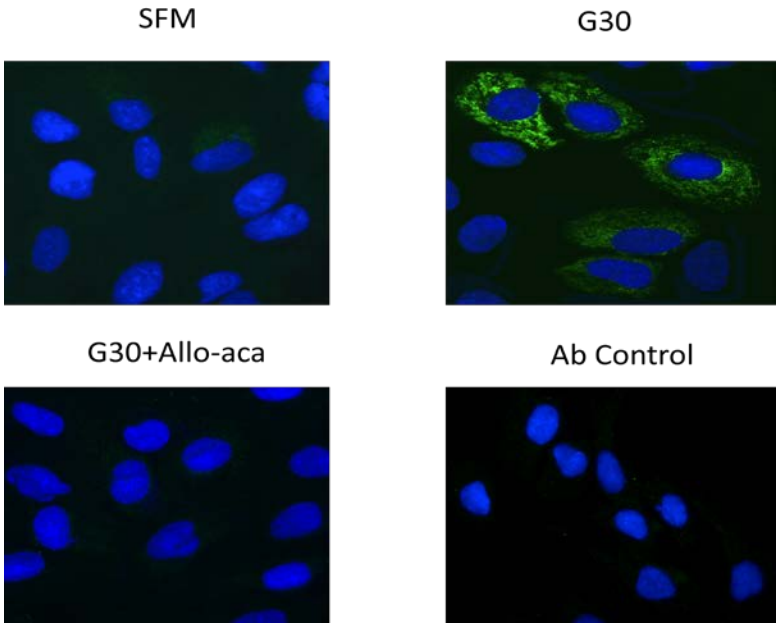

Figure 2. Effects of high glucose and Allo-aca on leptin protein expression in RF/6A cells. Cells were synchronized in SFM and placed in G30 medium for 72 h in the presence or absence of 250 nM Allo-aca (G30+Allo-aca). The expression of leptin protein (green immunofluorescence) was detected with specific Abs while cell nuclei (blue fluorescence) were detected with DAPI, as described in Materials and Methods. Leptin Ab control is represented by G30-treated cells stained with the secondary Ab only.

- **Hyperglycemia induces migration of RF/6A cells through ObR-dependent mechanism.**

HG as well as leptin are known to induce endothelial cell migration, however it is not known if these two pathways are integrated. Here, we tested the effects of G30 and leptin on directional

cell migration using a wound-healing assay and then examined the requirement of ObR activation in G30-induced migration. As expected, RF/6A cells exhibited basal migration capabilities in SFM and in mannitol, likely due to the presence of autocrine leptin and other motogenic factors [57] (**Figure 3**). G30 exposure accelerated cell migration by 60% and 74% vs. SFM and basal conditions, respectively ($p<0.05$). Similarly, greatly increased migration (61% and 75% vs. SFM and basal conditions, respectively, $p<0.05$) was noted in cultures treated with 250 ng/mL leptin (**Figure 3**). Notably, in the presence of 100 and 250 nM Allo-aca, the effects of G30 and leptin were reduced to SFM levels or below (**Figure 3** and data not shown).

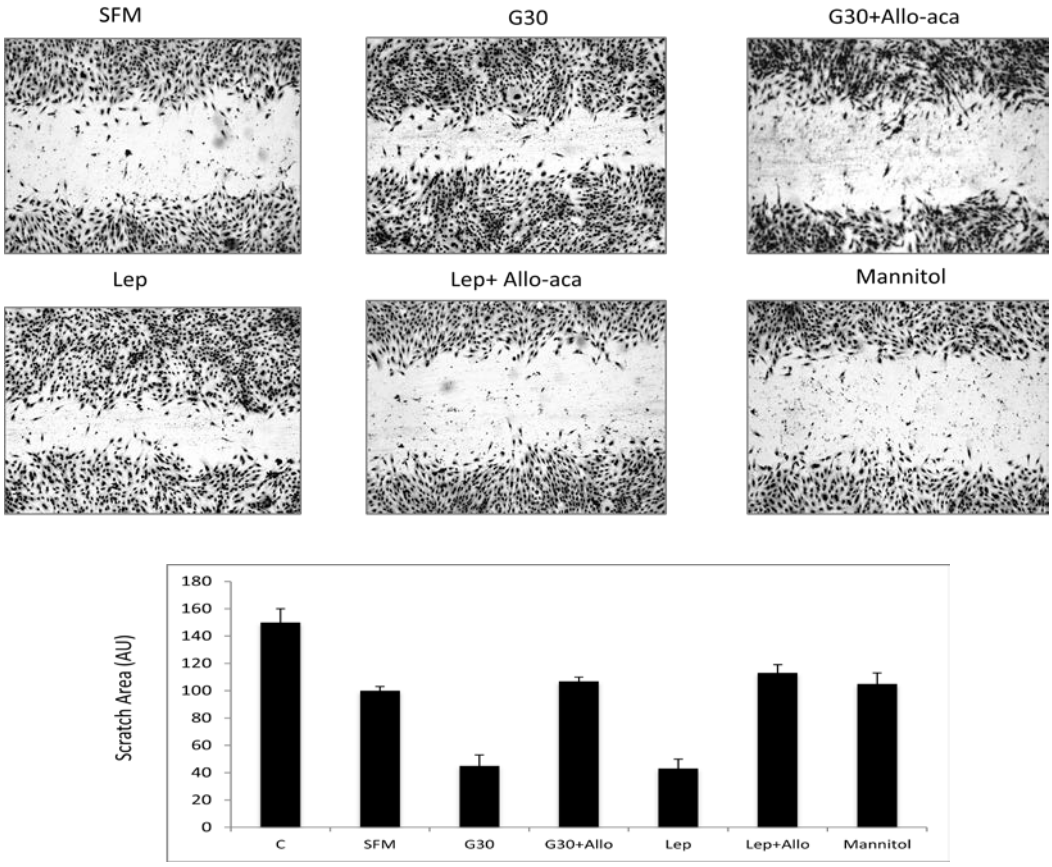

Figure 3. Effects of high glucose, leptin, and Allo-aca on migration of RF/6A cells. Wounds were produced in confluent RF/6A cell cultures, as described in Materials and Methods. Immediately after wounding, the cells were photographed and then shifted for 24 h in SFM, G30 medium (G30) with or without 250 nM Allo-aca (G30+Allo-aca), SFM containing 250 ng/ml leptin (Lep) with or without 250 nM All-aca (Lep+Allo-aca), or SFM containing 30 mM mannitol. The scratch areas before and after treatments were measured as described in Materials and Methods and are shown in the graph. Statistically significant changes ($p\leq0.05$) vs. SFM are marked with asterisk.

- **ObR activity is required for hyperglycemia- and leptin-dependent chemotaxis and chemokinesis of RF/6A cells.**

The involvement of the leptin pathway in G30-dependent migration was validated using Boyden chamber assays. In this assay, exposure to G30 stimulated chemokinesis of RF/6A cells by ~36%, and the presence of 50-250 nM Allo-aca reduced cell migration to approximately basal levels or below (**Table 1**). Furthermore, leptin at 50-250 ng/mL concentrations activated chemokinesis and chemotaxis of RF/6A cells. The greatest chemotactic and chemokinetic cell activity (95% and 66%, respectively) was observed under 100 ng/mL leptin treatment (**Table 2**). This chemotactic and chemokinetic response was significantly reduced in the presence of 50-250 nM Allo-aca by 65-90% and 40-92%, respectively ($p<0.05$) (**Table 3**).

Table 1. Effects of HG on RF/6A cell migration.

Treatment	Migrating Cells (% vs. SFM)
G30	+36 ±4
G30+Allo-aca 50 nM	-6 ±1
G30+Allo-aca 100 nM	-10 ±0
G30+Allo-aca 250 nM	-17 ±3

G30 medium with or without Allo-aca wasplaced in both chambers. Migration at 24 h is expressed as percentage increase or decrease ± SD vs. the values in SFM, as described in Materials and Methods.

Table 2. Chemotactic and chemokinetic effects of leptin in RF/6A cells.

Treatment	Migrating Cells (% vs. SFM)
Leptin 50 ng/mL, lower chamber	**+ 72±5**
Leptin 100 ng/mL, lower chamber	**+ 94±4**
Leptin 250 ng/mL, lower chamber	**+ 71±7**
Leptin 50 ng/mL, both chambers	**+ 59±7**
Leptin 100 ng/mL, both chambers	**+ 66±5**
Leptin 250 ng/mL, both chambers	**+ 58±8**

SFM containing different concentrations of leptin was placed either in lower chamber only or in both chambers. The cells were plated in the upper chamber. Migration was assessed after 24 h and expressed as percentage increase or decrease ± SD vs. the values in SFM, as described in Materials and Methods.

Table 3. Effects of Allo-aca on chemotactic and chemokinetic activity of leptin in RF/6A cells.

Treatment	Migrating Cells (% vs. SFM)
Leptin 100 ng/mL+Allo-aca 50 nM, lower chamber	**+ 33±4**
Leptin 100 ng/mL+Allo-aca 100 nM, lower chamber	**+ 15±3**
Leptin 100 ng/mL+Allo-aca 250 nM, lower chamber	**+ 9±2**
Leptin 100 ng/mL+Allo-aca 50 nM, both chambers	**+ 40±3**
Leptin 100 ng/mL+Allo-aca 100 nM, both chambers	**+ 13±2**
Leptin 100 ng/mL+Allo-aca 250 nM, both chambers	**+ 5±1**

SFM containing 100 ng/mL leptin and different concentrations of Allo-aca was placed either in lower chamber only or in both chambers, while the cells were plated in the upper chamber. Migration at 24 h is expressed as percentage increase or decrease ± SD vs. the values in SFM, as described in Materials and Methods.

- **Hyperglycemia stimulates RF/6A endothelial cell differentiation through ObR-dependent mechanism.**

Next, we tested whether or not ObR activation is necessary for HG-dependent endothelial

cell differentiation. Using in vitro angiogenesis (tube formation) assays, we found that G30 exposure increases the number of ES by ~40% and the presence of 100 or 250 nM Allo-aca totally blocks this effect ($p<0.05$) (**Figure 4** and data not shown).

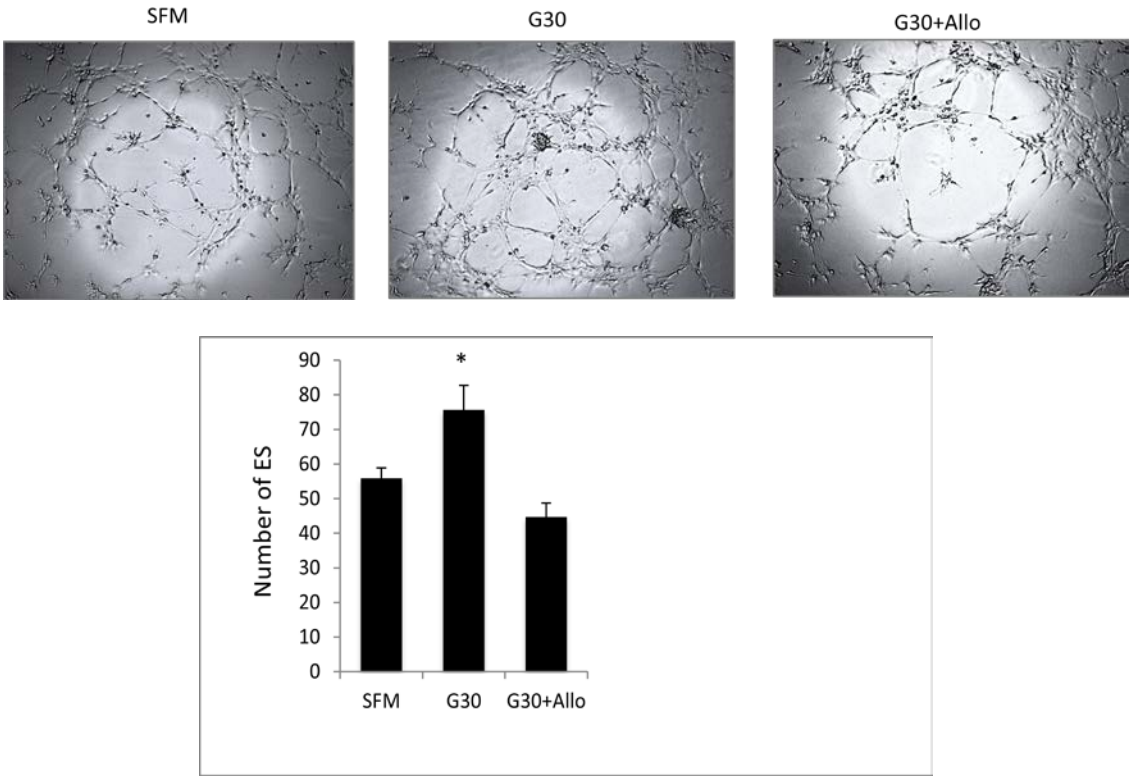

Figure 4. Effects of high glucose on angiogenic differentiation of RF/6A cells The angiogenic assays were carried out as described in Materials and Methods. Untreated cells (SFM) and cells pretreated with high glucose medium (G30) with or without 250 nM Allo-aca (G30+Allo-aca) were cultured for 3 h and photographed. The pictures represent ES formation under different treatments. The graph shows the number of ES (±SD) per visual field. Statistically significant changes ($p\leq0.05$) vs. SFM are marked with asterisk.

- **Hyperglycemia does not influence the growth of RF/6A cells.**

Exposure to HG has been reported to either induce or not affect the proliferation of retinal endothelial cells [42–44]. Here we assessed using direct cell counting if G30 can influence RF/6A cell growth. In several experiments, no mitogenic effects of G30 treatment at 24-96 h were observed. The maximal increase in cell number (~4%) that was noted at 72 h was not statistically significant vs. SFM conditions.

- **Several hyperglycemia-induced intracellular responses in RF/6A cells are mediated through ObR.**

HG as well as leptin has been shown to induce several intracellular pathways in endothelial cell models [40,42,43,45,57,86,96]. We studied, using retinal endothelial cells RF/6A, the effects of 24-96 h G30 exposure on the activation and/or expression of several key molecules implicated in angiogenic differentiation, inflammatory and stress response, and examined the involvement of ObR in these molecular responses (**Figure 5 A and B**).

First, we found that hyperglycemia increases ObR expression in RF/6A cells at 24-96h. The maximal ObR expression was found at 96 h of G30 exposure (~96% over untreated cells, $p<0.05$) (**Figure 5A**). In parallel, we found that G30 treatment and ObR upregulation coincided with the activation of STAT3 (max 36% at 96 h), ERK1/2 (max 62% at 72 h), Akt (max 87% at 72 h), and AMPK (max 69% at 96 h), as measured by phosphorylated/total protein ratios. Several of the above HG-dependent responses were decreased or modified in the presence of 100 nM Allo-aca. Specifically, Allo-aca decreased G30-induced phosphorylation of STAT3 at 72 h (~30% at 72 h), ERK1/2 (~70% and ~30% at 72 and 96 h, respectively), and Akt (~70% and 30% at 72 h and 96 h, respectively). In addition, Allo-aca decreased G30-dependent upregulation of ObR by ~30% at 96 h. However, Allo-aca did not modify the levels of phosphorylated AMPK (**Figure 5A**).

Furthermore, in RF/6A cells, G30 increased the expression of GRP at 24-96 h (max 95% at 96h), COX2 at 24-72 h (max 128% at 72h), and PAI-2 at 72-96 h (max 37% at 96h) (**Figure 5B**). On the other hand, the long-term exposure to HG resulted in significantly decreased expression of HIF-1α, CREB, and pPERK (**Figure 5B**). The presence of Allo-aca counteracted G30-dependent HIF-1α downregulation at 96 h, and COX2 upregulation at 24-72 h. In addition, in the presence of Allo-aca, PAI-2 expression was increased by 53% vs. G30 conditions at 96 h (**Figure 5B**).

Figure 5 A. Effects of high glucose and Allo-aca on intracellular signaling in RF/6A cells.

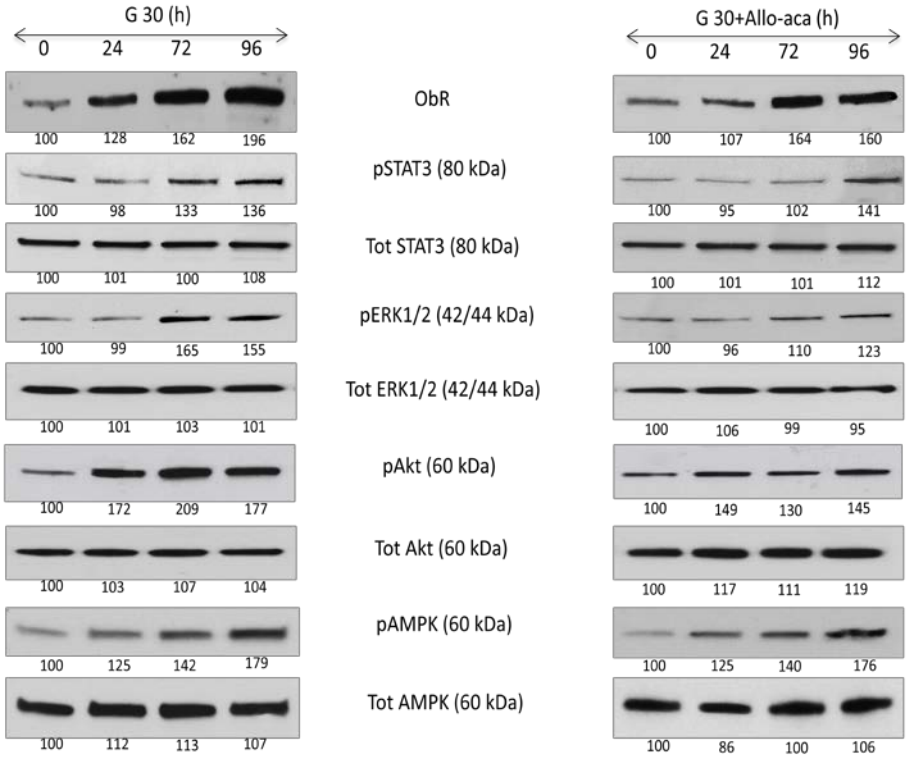

Figure 5 B. Effects of high glucose and Allo-aca on intracellular signaling in RF/6A cells.

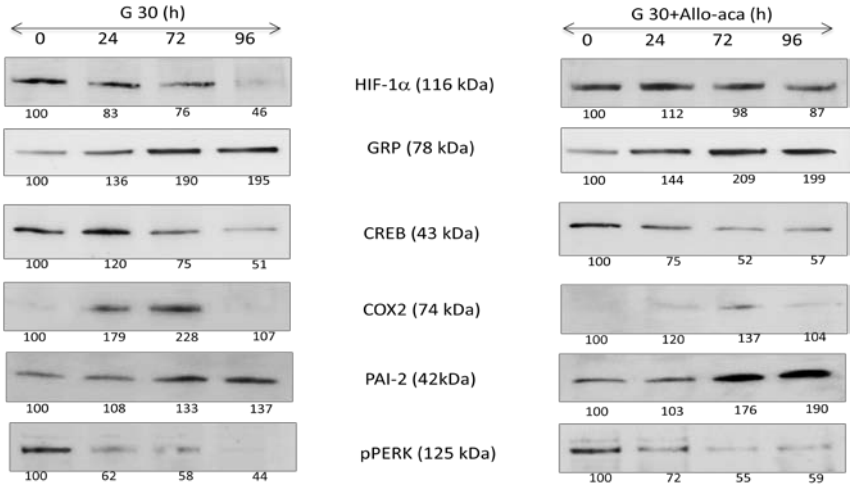

Figure 5 A and B. Effects of high glucose and Allo-aca on intracellular signaling in RF/6A cells. Cells were stimulated with high glucose medium (G30) with or without 250 nM Allo-aca (G30+Allo) for 24, 72 and 96 h and the expression of phosphorylated (p) and total (Tot) proteins was determined by WB and quantified, as described in Materials and Methods. The levels of β-actin were assessed as control of loading. The numbers under WB panels represent relative densitometry values (%) of phosphorylated and total proteins, with the value in SFM is taken as 100%. The representative blots of at least 3 experiments are shown.

DISCUSSION

It has been recognized that leptin promotes neovascularization and angiogenesis via direct vascular mechanism [70, 82, 83]. A recent study has revealed that plasma and vitreous leptin concentrations are correlated with the degree of DR [68,70,72]. These observations suggest that leptin may be involved in the development of DR [70].

There is also evidence that leptin indirectly modulates angiogenic responses induced by fibroblast growth factor-2 (FGF-2) and VEGF. Leptin and FGF-2 or VEGF could produce synergistic effects in stimulation of blood vessel growth in tissues where these factors are coexpressed [67]. The signaling pathways stimulated by leptin in endothelial cells support its angiogenic synergism with FGF-2 and VEGF. Leptin stimulated angiogenesis is mediated through the Jak-STAT pathway, whereas VEGF-induced angiogenesis is most likely mediated by the protein kinase C pathway, and FGF-2 induced angiogenic response is transduced by mitogen-activated protein kinases-regulated pathways [67,82,97].

We and others reported previously that leptin activated STAT3 in retinal endothelial cells. We found that in two ophthalmic cell models 6 or 12 h leptin stimulation increased STAT3 phosphorylation [57]. Suganami et al. reported that the leptin-induced VEGF mRNA expression was abolished by adenoviral transfection of dominant-negative STAT3 [70]. These observations, suggest that leptin increases VEGF mRNA expression in retinal endothelial cells by STAT3 activation. This notion is consistent with the presence of STAT3 binding sites in the 5' flanking region of the human VEGF gene [70,98]. Since VEGF plays a critical role in the proliferation of retinal endothelial cells [70, 99, 100], it is likely that leptin stimulates retinal neovascularization through the upregulation on endothelial VEGF and/or synergistically with VEGF [67,70].

HG has been implicated in the activation of numerous key mechanisms/pathways, including oxidative and nitrative stress [42,101,102], advanced glycation and products [103], and aldose reductase [104,

[105], which, in concert, contribute to retinal vascular dysfunctions and DR. Understanding how these pathways are activated and/or impact retinal vascular cell function under hyperglycemic conditions will aid in the development of new modalities to halt the development and progression of the disease.

HG exposure plays a crucial role in DR onset and progression [24,25]. However, the impact of HG on the activation of leptin/ObR axis in retinal endothelial cells has never been explored. In this study we demonstrated, for the first time, that HG exposure upregulates leptin and ObR expression, and modulates several ObR-dependent biological responses, i.e., migration, chemotaxis, chemokinesis, angiogenic cell differentiation and intracellular signaling. In addition, our data identified several HG-mediated biological effects that are dependent on ObR activation.

First, we observed that long-term HG treatment significantly increased leptin mRNA and protein in retinal endothelial cells. This is the first report linking HG to overexpression of the leptin/ObR system in ocular cell models. Our results are in line with three other reports that noted the sensitivity of the leptin/ObR system to HG in mammary epithelial cells [106], cardiomyoblasts [74], and neurons [75]. Interestingly, HG is known promote the synthesis of several angiogenic factors, including VEGF in human and rat retinal endothelial cells [44,45]. This is in line with our observations that HG increased VEGF mRNA levels in RF/6A cells, however this increase was significantly lower than that of leptin mRNA (50% vs. 300%) (data not shown). Our observation that HG potentiated retinal endothelial cell migration, is in agreement with Huang and Sheibani who found that the most significant impact of HG on retinal endothelial cells was enhanced migration. In addition, our results demonstrated that HG stimulated cell differentiation to create tube-like structures, consistent with other reports in human and mouse retinal endothelial cells [42,45]. In our model, the biological effects of HG were similar to that obtained with 25-50 ng/mL leptin [57].

The impact of HG on proliferation of retinal cells is unclear. We found that HG treatment was not mitogenic in RF/6A cells, which confirms the observations of Huang et al. in mouse retinal

cells [42] but stands in contrast with results from primary bovine microvascular cells and human retinal cells [43,44]. These discrepancies could be attributed to differences in cell models as well as the length of HG treatments and various methodologies of proliferation assessment. In addition, significant differences could exist in the amount and repertoire of cytokines synthesized under HG conditions in different cell lines. In our model, the amounts of leptin induced by HG are probably not sufficient to induce mitogenesis or some anti-mitogenic factors are produced in parallel.

The involvement of ObR-dependent signaling in HG-induced leptin expression and HG-dependent migration and angiogenesis was confirmed with the use of our ObR antagonist, Allo-aca at 100-250 nM concentrations. The specificity and efficacy of this peptidomimetic has been demonstrated before in many experimental models, including retinal endothelial cells [57,85,90,92,93].

HG has been shown to exert profound effects on a variety of biological functions through the activation of intracellular signaling pathways. In agreement with data obtained in different ophthalmic cell models, HG treatment activated or modulated several intracellular pathways in RF/6A cells. The activation of STAT3, Akt, ERK1/2 by HG has been shown before in different retinal endothelial cells [40,45,107].

It is know that oxidative stress induced by HG is an important pathway of DR complications [44]. Recent study showing that diabetic-induced retinal vascular dysfunction can be prevented by inhibitors of reactive oxygen species provide further support for the role of oxidative stress in DR [44, 108,109]. Oxidative stress has been correlated with the increased production of VEGF under in vitro conditions and is thought to be involved in the upregulation of VEGF expression during diabetes [44, 110].

Consistent with other reports, HG increased COX-2 expression [43] and modulated ER-stress related proteins such as GRP and pPERK [45]. We also noted a time-dependent activation AMPK in response to HG, which supports suggestions that activation of AMPK counteracts HG effects in endothelial cells [111–113].

HG is known to either increase or decrease HIF-1α expression in different models [49,50,52] and our findings suggest that long-term HG exposure can induce progressive destabilization of HIF-1α in retinal endothelial cells. HIF-1α is a key oxygen sensor and mediator that regulates multiple target genes, such as VEGF, which is a key pro-angiogenic factor in DR [51,114,115]. Over the past decade, almost all retinal cells have been shown to express HIF-1α and VEGF in DR [51]. Therefore, it is plausible that HIF-1α has critical roles in regulation of cellular functions not only under hypoxic conditions, but also in conditions such as metabolic disturbance and inflammation [50]. In addition, a fraction of HIF-1α target genes is superimposed on the genes dysregulated in the diabetic kidney, indicating a contribution of HIF-1α to the development of diabetic glomerulophaty thus not only in DR [50].

HG also induces inhibitors of matrix-degrading enzymes including plasminogen activator inhibithor-1 (PAI-1), which also leads to an accumulation of extracellular matrix proteins [50,116]. Our results demonstrated for the first time, that HG increases in a time-dependent manner the expression of PAI-2, a protein acutely upregulated in inflammation and cellular stress [117,118] and also implicated in regulating cell proliferation and turnover in the cornea and conjunctiva [119].

Our data suggested that the impact of HG on intracellular signaling is in part mediated through ObR activation. Specifically, using Allo-aca, we found that the ObR antagonist counteracted HG effects on STAT3, ERK1/2, Akt, COX2, and HIF-1α. In addition, Allo-aca further potentiated HG effects on PAI-2. However, some HG-induced pathways, including AMPK activation, and GRP and CREB expression appear to be ObR independent.

CONCLUSIONS

Our findings implicate the leptin/ObR axis in several biological effects elicited by HG on retinal endothelial cells, including the activation of angiogenic response. While leptin has been reported to stimulate ocular angiogenesis, our results strongly suggest that leptin could play a greater role in promoting neoangiogenesis under hyperglycemic than under euglycemic conditions. In this context, leptin signaling could represent a new target in DR and ObR antagonists could be developed as novel compounds for the treatment of ocular neovascularization.

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UNIVERSITA' DELLA CALABRIA
Dottorato di Ricerca in Biochimica Cellulare
ed Attività dei Farmaci in Oncologia.

Il giorno 27 Ottobre 2014 alle ore 11.30 si è riunito, per via telematica, il Collegio dei Docenti del Dottorato di Ricerca in Biochimica Cellulare ed Attività dei Farmaci in Oncologia per discutere il seguente ordine del giorno.

1. **Valutazione relazioni fine anno dottorandi XXVI, XXVII e XXVIII ciclo.**
2. **Varie ed eventuali.**

OMISSIS

1. **<u>Punto 1 all'ordine del giorno</u>: Valutazione relazioni fine anno dottorandi XXVI, XXVII e XXVIII ciclo.**

   Sono pervenute le relazioni di fine anno dei Dottorandi del XXVI, XXVII ed XXVIII ciclo. Tra queste anche le relazioni dei dottorandi con borsa FSE nell'ambito del Polo di Innovazione Regionale delle *"Tecnologie della Salute"*. Come ogni anno il Collegio dei Docenti è chiamato ad esprimere il proprio parere sul lavoro svolto dai dottorandi.
   Il presidente chiede al Collegio dei Docenti di esprimere un giudizio sulle relazioni presentate dai Dottorandi con borsa FSE. La Dott.ssa CIONE si astiene dal giudicare le relazioni di fine anno dei Dottorandi.

OMISSIS

Dott.ssa Coroniti Roberta. La Dott.ssa Coroniti ha svolto gli ultimi due anni di dottorato presso lo Sbarro institute for cancer research and molecular medicine della Temple University di Philadelfia (USA) sotto la guida della Dr.ssa Eva Surmacz interessandosi dei fattori legati all'obesità in relazione ad aspetti patologici quali retinopatie e cancro. Durante il triennio, la Dott.ssa Coroniti ha dimostrato piena dedizione lavorativa, notevole interesse nell'apprendimento e nell'elaborazione di numerose tecniche laboratoristiche, ottime capacità critiche nella scelta dei protocolli sperimentali e nella valutazione dei dati ottenuti. La dedizione e l'impegno mostrato per la ricerca hanno portato alla pubblicazione di due lavori su riviste internazionali. Pertanto il tutor propone che la Dott.ssa Coroniti venga ammessa all'esame finale per il conseguimento del titolo di dottore di ricerca.

Il Collegio dei docenti valutato il contenuto della relazione di fine anno della Dott.ssa Roberta Coroniti esprime parere favorevole alla partecipazione all'esame finale del corso di Dottorato a cui si astiene la Dott.ssa CIONE.

OMISSIS

Rende, 27.10.2014

Il Segretario
Dott.ssa Francesca De Amicis

Il Coordinatore
Prof. Diego Sisci

AL Magnifico Rettore
Università della Calabria

Presentazione al Collegio dei docenti della Dott.ssa Roberta Coroniti per il conseguimento del titolo di "Dottore di Ricerca in Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XXVII ciclo)

La Dott.ssa Roberta Coroniti ha svolto il corso di Dottorato di Ricerca in "Biochimica Cellulare e ed Attività dei Farmaci in Oncologia" (XXVII ciclo) presso il Dipartimento di Farmacia e Scienze della Salute e della Nutrizione, ex Farmaco-Biologico dell'Università della Calabria ed ha partecipato alle attività seminariali e didattico-formative organizzate dallo stesso Dipartimento.
Durante tale periodo si é interessata principalmente allo studio dei meccanismi molecolari coinvolti nella regolazione, da parte degli estrogeni, di specifiche funzioni legate alla progressione della patologia tumorale mammaria, approfondendo le sue conoscenze teoriche mediante lo studio costante della letteratura corrente. In particolare la Dott.ssa Coroniti ha studiato i meccanismi alla base dei processi di metastatizzazione ponendo in relazione l'espressione e funzione della proteina Forkhead box class O3a (FoxO3a) con l'acquisizione del fenotipo metastatico in cellule di carcinoma mammario. I risultati ottenuti hanno evidenziato una dicotomia d'azione dipendente dall'espressione del Recettore Estrogenico α (ERα). L'over-espressione di FoxO3a causa una drastica riduzione della motilità e delle capacità invasive, inoltre, riduce la crescita cellulare in assenza di adesione di cellule di cancro positive per l'espressione di ERα. Effetti opposti sono evidenziabili in cellule tumorali ERα negative. Questi dati dimostrano che ERα è un fattore fondamentale nel determinare le funzioni attivatorie o inibitorie della proteina FoxO3a nel controllo dell'aggressività delle cellule tumorali. Inoltre, lo studio ha messo in luce uno dei possibili meccanismi attraverso il quale si realizza tale funzione dimostrando che, in cellule esprimenti ERα, gli eventi mediati da FoxO3a sono direttamente correlati ad un incremento dell'espressione della proteina Caveolina 1 (Cav1), un costituente essenziale delle caveole che è noto essere correlato negativamente sia all'invasività tumorale che alla formazione di metastasi. Tale aumento si ottiene attraverso il legame di FoxO3a a specifiche regioni regolatrici della trascrizione del gene Cav1 che è dipendente dall'espressione di ERα. Lo studio è stato condotto anche in vivo, avvalendosi della collaborazione dell'U.O. di Anatomia Patologica dell'Ospedale Civile dell'Annunziata di Cosenza che ha fornito le sezioni di tessuto tumorale positive e negative per ERα di cancro mammario invasivo e di carcinoma duttale in situ. L'analisi immuno-istochimica ha mostrato che il ruolo di FoxO3a non dipendente dalla sua espressione quanto dal comparto cellulare che occupa. Esso è

nucleare nei tumori non metastatici ERα positivi, mentre è sempre più citoplasmatico in quelli invasivi ERα negativi. Il lavoro evidenzia nuovi aspetti nella relazione funzionale tra ERα e FoxO3a indicando, quest'ultimo, come possibile bersaglio terapeutico sia in tumori ERα positivi che negativi.

Dal secondo anno di Dottorato la Dott.ssa Coroniti ha iniziato il suo stage presso il laboratorio di Obesity and Cancer diretto dalla Prof.ssa Eva Surmacz presso lo Sbarro Institute for Cancer Research and Molecular Medicine della Temple University di Philadelphia, USA. In questo laboratorio è rimasta fino al termine del suo percorso formativo svolgendo anche il lavoro di tesi. Durante il periodo di stage ha studiato i processi di neo-vascolarizzazione oculare e di crescita cellulare indotti dalla leptina in cellule endoteliali della retina e della cornea. In particolare, lo studio si è concentrato sugli effetti angiogenici indotti dalla leptina in condizioni di iperglicemia-indotta. Il risultato dell'azione pro-angiogenica della leptina, nel modello oftalmico trattato con alte concentrazioni di glucosio, ha mostrato una elevata capacità di stimolare tutti i processi coinvolti nella migrazione e differenziazione angiogenica. Inoltre, durante la stessa condizione di iperglicemia-indotta, nelle cellule è stato notato un notevole incremento dell'espressione dei livelli di mRNA e proteine, della leptina e del suo recettore. Questi effetti, coincidevano con l'attivazione di diversi segnali intracellulari, alcuni implicati nella risposta angiogenica e metabolica, altri coinvolti nello stress ossidativo e nella regolazione dei livelli di proteina in risposta al glucosio ed alcuni mediatori dell'infiammazione. Di notevole interesse è stata la valutazione delle funzioni inibitorie sull'azione della leptina svolte da uno specifico antagonista del recettore per la leptina, Allo-aca. Infatti, tutti i processi pro-angiogenici ed i segnali intracellulari implicati, venivano parzialmente o totalmente bloccati dalla presenza di Alloaca (ObR-antagonista), un peptide, in grado, in vitro, di riconoscere e legare ObR, e di contrastarne gli effetti anche a basse concentrazioni. Questi risultati, per la prima volta, hanno dimostrato come l'asse leptina/ObR, sia coinvolto negli effetti biologici indotti dalle alte concentrazioni di glucosio, nelle cellule endoteliali oftalmiche. Questa via potrebbe rappresentare, perciò, un importante target terapeutico nel trattamento della neovascolarizzazione oculare.

La Dott.ssa Coroniti durante l'intero percorso formativo di dottorato ha dimostrato entusiasmo e notevole attitudine alla ricerca. Dotata di spirito critico nella elaborazione e nella interpretazione dei risultati sperimentali, ha acquisito ottima padronanza delle metodologie e delle tecniche di biologia molecolare utilizzate. L'esperienza maturata si evince dalla capacità di svolgere attività di ricerca in autonomia e con senso di responsabilità.

Il contributo della Dott.ssa Coroniti è dimostrato dai lavori scientifici pubblicati sulle seguenti riviste internazionali:

1. Guan M, Romano G, Coroniti R, Henderson EE. Progress in oncolytic virotherapy for treatment of thyroid malignant neoplasm. J Exp Clin Cancer Res. 2014 Nov 1;33(1):91.
2. Scolaro L, Parrino C, Coroniti R, Otvos L Jr, Surmacz E. Exploring leptin antagonism in ophthalmic cell models. PLoS One. 2013 Oct 3;8(10):e76437.
3. Sisci D, Maris P, Cesario MG, Anselmo W, Coroniti R, Trombino GE, Romeo F, Ferraro A, Lanzino M, Aquila S, Maggiolini M, Mauro L, Morelli C, Andò S. The estrogen receptor α is the key regulator of the bifunctional role of FoxO3a transcription factor in breast cancer motility and invasiveness. Cell Cycle. 2013 Nov 1;12(21):3405-20.

Pertanto, si esprime parere estremamente positivo sull'attività scientifica svolta dalla Dott.ssa Roberta Coroniti.

Rende, 18-11-2014

Docente tutor e
Coordinatore del Dottorato
Prof. Diego Sisci

Journal of Experimental & Clinical Cancer Research

REVIEW Open Access

Progress in oncolytic virotherapy for the treatment of thyroid malignant neoplasm

Mingxu Guan[1,2*], Gaetano Romano[1,3], Roberta Coroniti[3] and Earl E Henderson[1]

Abstract

Thyroid malignant neoplasm develops from follicular or parafollicular thyroid cells. A higher proportion of anaplastic thyroid cancer has an adverse prognosis. New drugs are being used in clinical treatment. However, for advanced thyroid malignant neoplasm such as anaplastic thyroid carcinoma, the major impediment to successful control of the disease is the absence of effective therapies. Oncolytic virotherapy has significantly progressed as therapeutics in recent years. The advance is that oncolytic viruses can be designed with biological specificity to infect, replicate and lyse tumor cells. Significant advances in virotherapy have being achieved to improve the accessibility, safety and efficacy of the treatment. Therefore, it is necessary to summarize and bring together the main areas covered by these investigations for the virotherapy of thyroid malignant neoplasm. We provide an overview of the progress in virotherapy research and clinical trials, which employ virotherapy for thyroid malignant neoplasm as well as the future prospect for virotherapy of thyroid malignant neoplasms.

Keywords: Oncolytic virotherapy, Thyroid malignant neoplasm, Thyroid cell biomarker

Background

Thyroid malignant neoplasm (TMN) occurs in the follicular or parafollicular thyroid cells. Thyroid, a butterfly-shaped gland located at the base of neck, produces hormones that regulate heart rate, blood pressure, body temperature and weight. Thyroid malignant neoplasm is the most common endocrine malignancy, accounting for 1.9% of all new malignant tumors diagnosed annually in the United States [1]. TMN can be classified according to their histopathological characteristics. Papillary (PTC), follicular (FTC), and anaplastic thyroid carcinomas (ATC) arise from endodermal-derived follicular cells, which represent the most abundant cellular population of the thyroid gland. PTC comprises 80% to 85% of all thyroid neoplasms, which is frequent in young females with excellent prognosis. FTC is accounting for approximately 10% to 15% of cases. PTC and FTC will lie in the low-risk group, have a low recurrence rate (<5%) and excellent survival rate (>98%) [2]. The least common histotype is ATC (1–2%), which has a rapid progression and a very poor prognosis with a mean survival time of 2–6 months. Surgery, radiotherapy and chemotherapy do not improve survival [3]. Medullary thyroid carcinoma (MTC), about 3% of all thyroid cancer cases, originates from parafollicular C cells, which produce the hormone calcitonin. Currently, the most effective management of aggressive thyroid malignant neoplasm is surgical removal of the thyroid, followed by radioactive iodine ablation and TSH-suppression therapy. However, more effective therapies are urgently needed to minimize dismal outcomes for the thyroid malignant neoplasm [4]. Oncolytic virotherapy is a promising anti-cancer strategy, because it can provide a local regional control, or eradication of tumors without cross-interference with standard therapies [5]. Oncolytic viruses can be divided into wild type viruses that are naturally oncolytic such as reovirus, measles virus, Newcastle disease virus (NDV), myxoma virus (MYXV), and vesicular stomatitis virus (VSV) and those that require genetic modification for selective oncolysis. Genetic modification for selective oncolysis involves either the insertion or deletion of genes [6-9].

In this review, we have recapitulated the important findings that were obtained in preclinical and clinical trials for the treatment of TMN, as well as the future goals, with a particular focus on the strategies in the design of virotherapy.

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Virotherapy for TMN

The viruses used for TMN mainly include three DNA viruses (Adenovirus, Herpesvirus and Vaccinia virus) and five RNA viruses (Reovirus, Newcastle disease virus, Vesicular stomatitis virus, Measles virus and Retrovirus). These viruses have been studied as potential therapeutics for monotherapy, or combination therapy in conjunction with chemotherapy and/or radiation therapy in pre-clinical or clinical trials (Table 1). We discuss these viruses in the order of the clinical study progress for TMN treatment.

Adenovirus - preclinical and clinical studies

Adenoviruses (Adv) are medium-sized (90–100 nm), nonenveloped viruses with an icosahedral nucleocapsid containing a double stranded DNA (dsDNA) genome between 26 and 48 Kb. This allows the virus to encode for 22 to 40 genes [10]. The two currently established receptors are CD46 for the group B human adenovirus serotypes and the coxsackievirus adenovirus receptor (CAR) for all other serotypes. A co-receptor is called αv integrin that results in endocytosis of the virus particle via clathrin-coated pits [11]. In this process, a series of viral proteins co-operate to promote efficient replication of the virus and its release. These major viral proteins include E1A, E1B-55kD, E1B-19kD, E3-11.6 kD and other associated proteins [12]. Human adenovirus have 57 distinct adenoviral serotypes have been found to cause a wide range of illnesses, from mild respiratory infections in young children to life-threatening multi-organ disease in people with a weakened immune system. Nearly all adults have antibodies to endemic serotypes 1, 2, 5, or 6 [13].

Various Adenovirus strains have been explored extensively as a viral vector for gene therapy and also as an oncolytic virus. A common strategy in adenovirus work is to express an enzyme inhibitor, a suicide gene such as express herpes simplex virus (HSV) thymidine kinase (tk) and then administer ganciclovir (GCV), or express p53, interleukin-2 (IL-2) and interleukin-12 (IL-12), or human sodium iodide symporter (NIS) or pigment epitheliumderived, factor (PEDF) as a potent inhibitor of tumor angiogenesis [14] and then used in combination therapy. Combination approaches have been explored as means to promote adenovirus replication and killing at tumor sites.

Shen et al. [15] constructed an oncolytic adenovirus with a Survivin targeted small hairpin RNA and a reporter gene (ZD55-Sur-EGFP). The virus can replicate specifically in cancer cells, reduce effectively both the expression of mRNA and protein expression of Survivin ($P < 0.0001$), induce cancer cell apoptosis and inhibit human colon adenocacinoma SW480 cell line growth both in vitro and in vivo [15]. Minemura *et al.* [16] characterized two adenovirus-derived vectors, which were combined with GCV to inhibit the growth of MTC cells. These two adenoviral-based vector systems were termed AdCMVtk and AdDCTtk. The latter contains human CALC-I minigene under the control of the CALC-I promoter, whereas the former carries the HSVtk gene driven by the human cytomegalovirus promoter. All cell lines transduced with AdCMVtk were rendered sensitive to GCV. Cell killing was also observed in HeLa, HepG2, rat MTC and TT (human MTC) cells by the combination of GCV and AdDCTtk vector [16]. Zhang *et al.* [17] evaluated cell-specific killing activity of replication defective adenovirus transducing TK gene under the control of the rat thyroglobulin (rTg) promoter (AdrTgtk) and then to transduce various cell lines. When FRTL-5 cells were infected with AdrTgtk followed by GCV treatment, more than 90% were killed [17]. Moreover, the enhanced

Table 1 Oncolytic viruses in TMN clinical trials

Virus	Genome	Genetic alterations	Delivery	Combination	Phase	References
Adenovirus	dsDNA	P53 (rAD-p53)	IT	Radioactive iodine/ Surgery	IV	24
		P53 (ONYX-015)	IT	Cisplatin/5-fluorouracil	Marketed	27
		Fas-c (VB-111)	IV	Anti-angiogenic agent	II	29
Reovirus	dsRNA	Natural strain (RT3D)	IT	Low-dose radiation/	II	35
			IT/IV	Gemcitabine/Carboplatin/ Paclitaxel/Docetaxel	III	37. 38
Herpes simplex virus	dsDNA	N/A				
Vaccina virus	dsDNA	N/A				
New castle disease virus	ssRNA	N/A				
Vesicular virus	ssRNA	N/A				
Measles virus	ssRNA	N/A				
Retrovirus	ssRNA	N/A				

Abbreviation:
IT, Intra-tumor injection.
IV, Intra-venous injection.

effect of combined HSV-tk and ganciclovir with IL-2 [18] and combined HSV-tk/GCV, IL-12 [19] and p53 [20] have also been evaluated. The tumor suppressor p53 is a transcription factor that regulates cell cycling, DNA repair and apoptosis. However, p53 mutations are not frequent in thyroid cancer. The exogenous wild type p53 could increase chemo sensitivity in three anaplastic thyroid carcinoma cell lines (BHT-101, SW-1736, and KAT-4), which had nonfunctional p53 and ATC cell lines have been observed [21]. It is probable that the majority of thyroid cancers activate an alternative pathway that compromises the function of wild-type p53. Well-differentiated thyroid cancers generally do not express a mutation in p53 [22].

Clinical trials were carried out using recombinant adenoviral-encoded human p53 gene (rAd-p53), combined with intensity-modulated radiation therapy (IMRT) for treatment of 46 patients with historically-diagnosed stage IV PTC. The patients received intratumoral injection. A month later, 76% (19/25) of patients underwent radical surgery. One-year survival rate was 96% and no local recurrence and distant metastases were observed in the cohort of patients with surgery [23]. Currently, the clinical trials, rAd-p53 gene therapy for advanced malignant thyroid tumors (stage III/IV) is in phase IV [24]. When ONYX-015 was combined with the standard chemotherapeutic agents cisplatin and 5′-fluorouracil to combat TMN, a considerable efficacy was reported in a proportion of cases [25]. ONYX-015 proved safe at a dose up to 2×10^{12} viral particles [26]. So far, ONYX-015 is the only oncolytic virus product on the market [27]. E1A-defective adenovirus dl922-947 and DeltaE1B55K (dl1520) mutants were compared in human thyroid anaplastic carcinoma cells in vivo and in vitro. The efficacy of dl922-947 exceeded that of dl1520 in all tested anaplastic thyroid carcinoma cells. The combination of dl922-947 with bevacizumab reduced significantly tumor growth compared with single treatments alone [28]. Recently, an important genetically modified antiangiogenic adenovirus, termed VB-111, exhibited to have significant antitumor activity for thyroid cancer. VB-111 expresses Fas-c in angiogenic blood vessels. VB-111 treatment resulted in 26.6% (P =0.0596), 34.4% (P =0.0046), and 37.6% (P =0.0249) inhibition of tumor growth in follicular, papillary and anaplastic thyroid cancer cell line models, respectively. No toxicity was observed in these studies [29]. The clinical trial based on VB-111 for oncolysis is currently underway for patients bearing thyroid malignant neoplasm. The multi-dose VB-111 phase II clinical trial dealt with the treatment of patients with gliobastoma multiforme, differentiated thyroid cancer and ovarian cancer (NCT0120506).

Reovirus - preclinical and clinical studies

Mammalian orthoreoviruses (reoviruses) are ubiquitous viruses that infect cells in the respiratory and enteric tracts. Reoviruses have a 70-85 nm diameter and are non-enveloped nearly spherical icosahedral particles. The icosahedral capsid is composed of an outer and inner protein shell. The double stranded RNA (dsRNA) genomes contain 10–12 segments, which are grouped into three categories corresponding to their size: L (large), M (medium) and S (small). Segments range from approximately 1 Kb to 3.9 Kb and each segment encodes 1–3 proteins (the L segment encodes for λ proteins, the M segment encodes for μ proteins and the S segment encodes for σ proteins). Replication occurs in cytoplasm and viral particles begin to assemble in the cytoplasm 6–7 hours after infection [30]. The virus can enter the host cell via an unknown cell surface receptor. The receptor is thought to include sialic acid and junctional adhesion molecules (JAMs). Reovirus is not considered a human pathogen, due to the absence of obvious symptoms, or known clinical impact during natural or experimental infection [31]. The virus seropositivity approaches 100% in adults [32].

The reoviruses have oncolytic properties, which are a bonus for the development of reovirus-based therapies for cancer treatment. Ikeda *et al*. proved that reovirus was effective to inhibit the growth of human head and neck squamous cells in vitro and in vivo [33]. Moreover, the efficacy of reovirus can be enhanced by combination with chemotherapy. A triple reovirus, cisplatin and paclitaxel combination showed a significantly more effective targeting than reovirus combined with either cisplatin, or paclitaxel [34]. These studies were carried out in four head and neck cancer cell lines in vitro and in vivo. The in vivo studies consisted of subcutanoeus injection of four head and neck cancer cell lines in athymic nude mice [34]. A Phase II clinical trial investigated the antitumor effects of intratumoral administration of REOLYSIN® in combination with low-dose radiation in patients with advanced cancers. A total of sixteen patients with advanced cancer were enrolled in the trial. Of these, partial responses were observed in two patients (thyroid, ovarian), for a total disease control rate of 93% in the treated lesions [35]. RT3D was combined with carboplatin/paclitaxel in patients with advanced cancers. Thirty-one heavily pretreated patients received study therapy in the phase I/II studies [36]. In summary, of the current phase I/II studies, reovirus showed safety both as a single agent when administered intratumorally and intravenously, as well as in combination therapy, with multiple chemotherapeutics such as gemcitabine, carboplatin and/or paclitaxel, and docetaxel. Similar findings were observed with radiation. Reovirus was therapeutically formulated and used in a phase III registration study in metastatic squamous cell carcinoma of the head and neck [37,38].

Herpes Simplex Virus – 1 - preclinical and clinical studies

The Herpes Simplex Virus – 1 (HSV-1) virion ranges from 180 to 300 nm in diameter and comprise an outer-lipid bilayer envelope embedded with glycoproteins. Beneath the envelope lies the tegument layer, which contains proteins and enzymes important in early virus infection. The capsid contains the large dsDNA genome, consisting of 152,261 base pairs in length and with a base composition of 67% G + C. HSV-1 has approximately 90 open reading frames, allowing for the transcription of at least 84 unique proteins [39]. HSV enters into the cell by fusion of the viral envelope proteins. Virus initial interactions occur when a viral envelope glycoprotein C binds to a cell surface particle called heparan sulfate. A second glycoprotein, glycoprotein D, binds specifically to at least one of three known entry receptors [40]. These include HSV entry mediator, nectin-1 and 3-O sulfated heparan sulfate. Once bound to the mediator, gD interacts with viral glycoproteins H and L complex. In cells that are permissive for the propagation of this virus, the replication cycle is usually completed within 20 hours, releasing viral progeny through cell lysis. HSV-1 infection is extremely common and approximately 90% of adults have been exposed to the virus. The virus can be spread through contact with saliva. Symptoms of herpes simplex virus infection include watery blisters in the skin, or mucous membranes of the mouth, lips or genitals. As neurotropic and neuroinvasive viruses, HSV-1 persists in the body by becoming latent in the cell bodies of neurons. Some infected people may experience sporadic episodes of viral reactivation [41].

The strategies used in adenovirus work were also utilized in designing oncolytic HSV, such as expression of the tk in the tumor, followed by GCV administration, or other chemotherapeutic drugs, which are converted by tk into a cell-lethal metabolite in human thyroid carcinoma cell lines and in nude mice [42]. Soler *et al.* [43] evaluated a strategy for treatment of MTC, combining "suicide" and IL-2 gene therapies. Tumors were produced in Wag/Rij rats by orthotopic injection of the rMTC 6–23 cell line, and/or derivatives expressing the HSV1-TK gene (rMTC-TK). GCV, which is selectively transformed into a toxic metabolite by HSV1-TK, totally eradicated rMTC-TK tumors in 60% of the animals [43]. In a similar study, the expression of both the HSV-TK and the hIL-2 therapeutic genes in infected cells was high and persistent with time and showed that treatment with GCV led to more than 90% tumor growth inhibition and 100% inhibition transduced cells proliferation [44]. Moreover, successful safety and efficacy preclinical studies were conducted in NV 1023 and G207 cell lines, both in vitro and in animal models. Yu *et al*. [45] tested the oncolytic HSV (NV1023) against seven different thyroid cancers, including one papillary (NPA-187), one follicular (WRO82-1), one medullary (DRO81-1) and four anaplastic (DRO90-1, ARO, KAT-4C and KAT-18) cell lines. With the exception of WRO82-1 cell line, all other cell lines at MOI 5 demonstrated >95% infection in vitro at day 2. DRO81-1 tumors demonstrated partial response and all NPA-187 tumors in athymic nude mice completely regressed following the administration of a single dose [45]. A preclincial study utilized HSV NV 1023 strain, which expresses HSV glycoprotein D receptors nectin-1 and HSV entry mediator [46]. The HSV NV 1023 strain exhibited cytotoxicity greater than 85% in anaplastic, medullary and papillary cancers [46]. Another study used HSV G207 and NV1023 strains in combination with paclitaxel and doxorubicin to induce oncolysis in three human anaplastic thyroid cancer cell lines [47]. A dose-dependent cytotoxicity was observed for all four agents. Interestingly, G207 exhibited synergistic cytotoxicity, when combined with paclitaxel. A single intra-tumor injection (it) of G207 combined with biweekly intraperitoneal (ip) injection of paclitaxel injections in athymic nude mice bearing human anaplastic KAT4 cell line in a flank showed significantly reduced mean tumor volume [47].

The following clinical trials were not designed for the TMN treatment; however they may provide useful information about the employment of HSV as vector for virotherapy. SEPREHVIR (HSV-1716) was utilized in three clinical trials to treat 47 patients with glioma, 5 patients with melanoma and 20 patients with squamous cell carcinoma of the head and neck [48,49]. No toxicity attributable to the use of HSV1716 was reported in any of these clinical studies. A new phase I/IIa clinical study for the treatment of mesothelioma commenced in 2012 [48]. This trial is based on HSV-1 and was previously tested in a Phase I trial for other solid tumors, such as melanoma, liver cancer, pancreatic cancer, lung cancer (NCT01935453) and head and neck cancer (NCT00931931). Efficacy and Safety Study of OncoVEXGM-CSF compared to GM-CSF in patients with melanoma is ongoing (NCT00769704) [49].

Vaccinia Virus - preclinical and clinical studies

Vaccinia virus (VV) is a member of the genus Orthopoxvirus of the family of Poxviridae. VV is a large dsDNA virus whose entire life cycle takes place within the cytoplasm of host cells. Vaccinia intercellular mature virus (IMV) particles are brick-shaped, approximately 300 × 240 × 120 nm in size, with a lipoprotein shell surrounding a complex core structure [50]. The core structure contains a linear, ds DNA genome of approximately 192 kb associated with a number of virus-encoded proteins [51]. VV has inverted terminal repeats (ITRs) that are required for VV DNA replication. The 192-kb genome encodes some 200 genes that are largely non-overlapping. VV produces three forms of infectious particles: IMV, cell-associated

enveloped virus (CEV) and extracellular enveloped virus (EEV) with a different cell surface binding sites [50]. IMVs enter cells by fusion with the plasma membrane [52]. EEVs enter cells by endocytosis followed by low pH disruption of the EEV outer membrane and fusion of the released IMV with endosomal membranes [53]. It typically begins 1–2 h after infection and generates large numbers of genome copies per cell within hours of infection, of which half are ultimately packaged into infectious virions [54]. The host response to VV infection is multifactorial. Immediately after VV invasion, nonspecific mechanisms involving apoptosis induction, complement, interferons, cytokines and natural killer cells serve as the first-line host defense. [55]. Although neutralizing antibodies are also involved in host protection during VV infection, the cell-mediated immune responses are known to be particularly potent and may be most critical for viral clearance [56]. T helper 1 (Th1) immune response is mediated by antiviral cytokines such as IL-12, IL-18 and interferon (IFN)-γ, and plays a critical role in host defense [57,58]. Th2 immune response is mediated by IL-4 and IL-10 and may actually suppress the host response to vaccinia [59]. In addition, due to VV infection, the host cell shut-off is the abrogation of class I and class II major histocompatibility complex (MHC) molecule production and presentation, thereby leading to poor recognition of the virus by T cells [60]. At least two virus-encoded factors were shown to inhibit the complement cascade: C3L and B5R. VV encodes at least three proteins to inhibit premature cell death caused by apoptosis [61].

The majority of studies for TMN in oncolytic mutant vaccinia virus were designed to assess the viral oncolysis efficacy in thyroid cell lines and animal models of thyroid malignant tumors [62]. For example, vaccinia virus strain recombinant GLV-1 h68 infection was detected in all cell lines in 24 hours and increased in intensity at 36 hours in six ATC cell lines after inoculation [63,64]. In vivo studies showed an enhanced anti-tumor efficacy with one i.v. GLV-1 h68 injection followed by multiple i.p. injections of Avastin [65]. Expression of human NIS (hNIS) was also used in the VV work [66,67]. Vaccinia virus (GLV-1 h153) carrying hNIS was able to infect, replicate and kill all ATC cell lines. At a MOI of 1.0, GLV-1 h153 reached near 100% cytotoxicity in 8305c and FRO at day 5. The efficacy of the treatment was also observed in infected ATC xenografts [66,67]. In addition, GLV-1 h153 achieved more than 90% cytotoxicity in human gastric cancer cell lines and xenografts [68].

Currently, there are no virotherapy clinical trials based on VV for the treatment of TMN. However, the VV results obtained from several other trials are certainly interesting. The phase I clinical trial that utlized GL-ONC1 proved that the virus could safely be administered intravenously in patients with advanced solid tumors [69]. JX-594 (Pexa-Vec) is a VV engineered by addition of the GM-CSF gene and deletion of the thymidine kinase gene, which limits viral replication to cells with high levels of thymidine kinase, typically seen in cancer cells with a mutated RAS or p53 gene [70]. It has orphan drug designation from US FDA and EUMA for the treatment of hepatocellular carcinoma (HCC). Two phase I trials were concluded and a phase II trial for primary liver cancer, alone and in combination with sorafenib is starting. Both GL-ONC1 and JX-594 provided promising results in preclinical studies [71].

Newcastle disease virus - preclinical and clinical studies

Newcastle disease virus (NDV), also known as avian paramyxovirus-1 (APMV-1) and belongs to the genus Avulavirus of the family Paramyxoviridae. NDV is enveloped and spherical, with a diameter in the range of 150 nm and the genome consists of single stranded negative-sense RNA (ssRNA) that contains six open reading frames (ORF) and comprises 15,186 nucleotides. The six ORF encode the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin-neuraminidase (HN) and the large protein (L). At least one additional nonstructural protein (V) and possibly a second one (W) are generated by RNA editing during P gene transcription [72]. Virus infection is initiated by attachment of the virion to the surface of the target cell. Binding of the viral HN glycoprotein to sialic acid-containing cell surface proteins, which serve as receptors, triggers the F protein promoted fusion of the viral envelope with the plasma membrane of the host cell through a pH-independent mechanism [73]. NDV has only one serotype and causes Newcastle disease, which is a contagious bird disease. The virus is transmissible to humans and can cause mild conjunctivitis and influenza-like symptoms [74]. NDV replicate up to 10,000 times faster in human cancer cells than in most normal human cells. NDV is a naturally oncolytic virus that has been shown to be safe and effective for cancer therapy. In 1999, promising results were reported using an attenuated strain of the NDV [75].

Recombinant NDV is an effective oncolytic agent against a panel of tumor cell lines including thyroid, head and neck, and mouse model [76]. For example, NDV (F3aa)-GFP, a genetically modified fusogenic NDV, was used for the treatment of head and neck squamous cell carcinoma. Four cell lines were highly susceptible to viral cytotoxicity, with more than 75% of cells lysed at day 6 at MOI 0.1, whereas two other cell lines were partially susceptible. Oncolytic NDV was used in a number of in vivo studies for the effective target of various solid tumors [77,78].

So far, no clinical investigation has been initiated with NDV for TMN. However, four clinical trials have been

conducted in the United States studied the use of NDV oncolysates in patients with metastatic melanoma. Some positive results were found in these studies [79].

Vesicular stomatitis virus - preclinical and clinical studies

The vesicular stomatitis virus (VSV) is a single negative ssRNA virus. VSV is a member of the Rhabdoviridae family, which includes the rabies virus. The virus particle carries a single approximately 11-kb (–) RNA encoding five genes: the nucleocapsid protein N, the phosphor-protein P, the matrix protein M, glyco protein G and the large polymerase protein L. The G spikes on the outside of the virus particles will help to attach to other cells that can be infected. The VSV G protein mediates viral attachment either to Low-Density Lipoprotein (LDL) receptor (LDLR), or an LDLR family member present on the host cell. Following binding, the VSV-LDLR complex is rapidly endocytosed. It then mediates fusion of the viral envelope with the endosomal membrane [80]. The M protein is part of the inner membrane of the virus and is responsible for shutting down the host and the viral translation. VV replication occurs in the cytoplasm [81]. There are two distinct immunological classes of VSV recognized: New Jersey (NJ) and Indiana (IND). In humans, the incubation period may vary from 24 hours to 6 days but it is usually 3–4 days. VSV grows rapidly, is zoonotic and non-pathogenic to humans [82]. VSV only causes a flu-like illness in infected humans [82].

The efficiency of VSV to express either human or murine IFN-β, or a methionine deletion at residue 51 of the matrix protein was been observed in vitro and in vivo [83]. Currently, there is no clinical investigation that utilizes VSV for the treatment of TMN. A VSV-based phase I trial is currently underway for the treatment of patients with liver cancer (NCT01628640). In this clinical, VSV particles expressing human interferon beta will be administered into patients via intratumoral injection.

Measles Virus - preclinical and clinical studies

Measles virus (MV) is a single-stranded, negative-sense, enveloped RNA virus of the genus Morbillivirus of the family Paramyxoviridae. The measles virus has two envelope glycoproteins on the viral surface: hemagglutinin (H) and membrane fusion protein (F). These proteins are responsible for host cell binding and invasion. Three receptors for the H protein have been so far identified: complement regulatory molecule CD46, the signaling lymphocyte activation molecule (SLAM) and the cell adhesion molecule Nectin-4 [84]. Humans are the natural hosts of the virus. No animal reservoirs are known to exist. This virus is the cause of measles, an infection of the respiratory system. Approximately 500,000 persons with measles are reported each year in the United States [85]. The vaccine-attenuated strains of MV show a promising potential for cancer therapy [86]. There is a study that utilizes MV-Edm, which was modified to include the NIS gene to be combined with radiology treatment. This study demonstrated the oncolytic efficacy of MV-NIS in BHT-101 and KTC-3, ATC-derived cell lines and mouse model [87].

There is no MV clinical investigation for TMN yet. However, there are two phase I trials that are recruiting patients with other types of malignancies. One trial will studying the side effects and best dose of the MV derivative producing CEA (MV-CEA) to treat patients with recurrent glioblastoma multiforme (NCT00390299). Another MV-NIS phase I trial will study the side effects and the best dose of viral therapy to treat patients with recurrent or metastatic squamous cell carcinoma of the head and neck (NCT01846091).

Retrovirus - preclinical and clinical studies

The murine leukemia virus (MLV) is one of the simplest retroviruses. The virus has a spherical shape that has a diameter of 80 to 100 nm. The envelope of the virus is covered with glycoprotein spikes. The viral genome is a single stranded, linear, positive-sense RNA molecule of approximately 8,000 nucleotides and 4 ORF. The genome contains gag, pol, and env regions, which encode for structural proteins, enzymes including the RNA-dependent DNA polymerase (reverse transcriptase), and coat proteins, respectively [88].

A MLV-derived retroviral vector termed pMFGIL-2TKSN expresses two therapeutic genes, such as HSVtk and IL-2. This retroviral-based vector system exhibited enhanced tumor growth inhibition in animal models [89]. In addition, Barzon *et al.* [44] used a similar retroviral vector system and evaluated its efficiency in vitro and in vivo, following GCV treatment [90]. This study showed an 80% reduction of the size of differentiated thyroid carcinomas [44]. Two patients with end-stage anaplastic thyroid carcinoma were treated with direct intratumor injection of retroviral vector expressing the human IL-2 gene and the suicide gene tk of HSV 1, followed by GCV administration. The treatment was safe and demonstrated local tumor necrosis [91]. To date, the RV clinical investigation has not been reported for TMN. A phase I study will test the safety of different doses of the patients own immune cells, which will be genetically modified to help recognize and destroy the cancer cells for treatment of castrate metastatic prostate cancer (NCT01140373).

Consideration for the oncolytic virus design

Based on previous research, oncolytic virus replication in normal cells remains a concern and, therefore, the efforts should be focused on improving the oncolytic

viral tumor selectivity and specificity [92], as the specificity of oncolytic virus is a most important factor that would eventually determine the clinical safety and efficacy for the therapeutics. Specific targeting of tumour cell can be achieved by taking advantage of the fact that tumour cells have altered microenvironment, display certain tumour specific receptors and modified cellular pathways [93]. In fact, the specificity of oncolytic virus depends on the oncolytic virus tropism, which can be modified. For example, expression profiles of multiple microRNAs can reshape the tropism of MV, which could be safer and efficient for a high-dose systemic administration [94]. Indeed, viruses with altered tropism may be used for the targeting of tumor-specific surface biomarkers. Several TMN immuno-histochemical and molecular biomarkers have been proposed. Some of them might be useful for this purpose, or for diagnosis. Biomarkers for TMN comprise: RET, vascular endothelial growth factor receptor, hepatocyte growth factor receptor, epidermal growth factor receptor, ATP-binding cassette sub-family G member 2, cytokeratin 19 (CK19), galectin-3, oncofetal fibronectin (OnfFN), hector battifora mesothelial cell 1, CD44, CD56, CD63, CD113, CD133, lysosomal enzymes cathepsin B, cathepsin L and lysosomal dipeptidyl peptidase II [95-97]. The distribution of these biomarkers in TMN is variable. Demellawy *et al.* [98] reported that the CD56 positive in all the lesions and tumors except for PTC in all cases (100%). CD56 was negative in all PTC cases (100%). CK19 showed positive expression in PTC accounting for 85% of cases and in 26% of non-PTC lesions/tumors. P63 expression was in 70% of cases of PTC and was consistently absent in all the non-PTC cases [98]. Medullary thyroid carcinomas account for less than 5% of thyroid nodules and exhibit a specific circulating biomarker, i.e. calcitonin (CT). Another major issue is the relevance of oncogene mutation and signaling transduction in TMN. The genetic alteration described in PTC is the RET/PTC rearrangement, encoding for a transmembrane tyrosine kinase receptor. The incidence of RAS mutations in these different histotypes ranges from 0%–50% in PTC, 14%–62% in FTC and 0%–60% in ATC. The over-expression of the MET/HGF receptor is 100-fold higher in PTC than in FTC, ATC and MTC [99]. In conclusion, a panel of biomarkers was studied for TMN. However, there is no validated biomarker that is specific for TMN.

It has been previously reported that BRAF mutations and rearrangements of RET (RET/PTC) prevent the differentiation of thyroblasts into prothyrocytes, resulting in the generation of papillary carcinomas, whereas PAX8-PPARγ1 rearrangement prevents differentiation of prothyrocytes into thyrocytes, resulting in the generation of follicular tumors [100]. We understand that human thyroid ancestor cells or embryonic stem cells (ES) cell-derived, endoderm-positive cells have been shown to express several early thyroid markers. For example, anaplastic carcinoma (Tg -, OnfFN +) derives from thyroid stem cells, papillary carcinoma (Tg+, onfFN +) from thyroblast cells, and follicular tumor (Tg+, onfFN -) from prothyrocytes, respectively [101]. According to

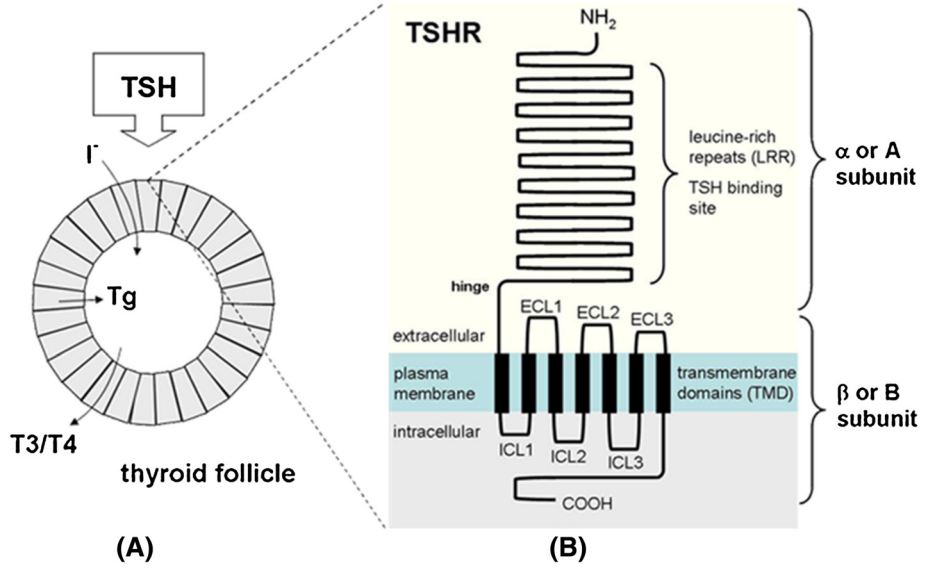

Figure 1 TSHR localization and structure. (A) Thyroid follicle, where the TSH-stimulated synthesis of thyroid hormone occurs following iodide uptake and organification into thyroglobulin. **(B)** TSHR protein structure, showing the β subunit composed of leucine-rich repeats and the N-terminus, and the β subunit composed of 7 trans-membrane domains, the intra-cellular and extra-cellular loops and the C-terminus. Figure adapted with permission from Atlas Genet Cytogenet Oncol Haematol [103].

this hypothesis, the thyroid tumors were caused by different mutations during thyroid fetal development. Thus, any event that prevents fetal thyroid cells from differentiation might stop carcinogenesis. Some thyroid specific biomarkers were expressed during normal human fetal development. Between E60 and E70, a series of events lead the thyroid primordial toward a functional thyroid gland able to produce and release hormones during the normal thyroid development in embryology [102]. The thyroid markers appear in that period. These include thyroglobulin (Tg), NIS, thyroid peroxidase (TPO), thyroid-stimulating hormone (TSH), and TSH receptor (TSHR) [102] (Figure 1) [103]. This information suggests that the thyroid markers, such as TSH, may be more useful for TMN virotherapy. TSH is a glycoprotein and consists of two subunits, the alpha (α) and the beta (β) subunit. The α subunit, a 92-amino acid sequence, is thought to be the effector region responsible for stimulation of adenylate cyclase (involved the generation of cAMP). The β subunit (TSHB), a 118-amino acid sequence, is unique to TSH, and therefore determines its receptor specificity [104,105]. The TSHR is mainly found on thyroid follicular cells. TSH binds TSHR with high affinity. TSHR is widely expressed in a variety of extrathyroidal tissues [102]. The level and the tissues distribution of TSHR and TPO are very similar to the distribution of NIS. Therefore, TSHR (or TPO) might still be considered as a fetal thyroid marker, which can be used for oncolytic virotherapy. Another strategy consists of expressing the Fab fragment of antibodies anti-TSHR, or anti-TPO, or anti-NIS. This might increase the oncolytic virus binding and/or entry specifically for cells expressing TSHR, or TPO, or NIS.

In addition, previous studies that utilized VSV and NDV as oncolytic agents provided encouraging results. Moreover, VSV and NDV are zoonotic pathogens. Humans have little to no immunity against these viruses, which facilitates their escape from host immune system. This allows for the oncolytic viruses to reach and kill malignant cells. Virtually, the final step to success is the selection of the site and route for virus delivery. Current studies have emphasized intratumoral delivery. The direct tumor injection cannot kill the tumor cells in blood stream, which may cause the tumor recurrence. Therefore, the systemic delivery through the bloodstream to reach cancer cells will be necessary. Studies are ongoing to improve efficient delivery of oncolytic viruses into the host. To this end, preclinical studies are focusing on the use of cellular carriers of oncolytic viruses [106]. The cellular carriers may shield oncolytic virus from neutralizing antibodies during delivery, providing a simple and effective tool to enhance the therapeutic efficacy of oncolytic viruses [106]. However, as a human therapeutic agent, the maximum purity that related with agent's safety and efficacy is very important. An improper agent preparation might cause some unacceptable adverse events. Therefore, it is important to improve the safety of therapeutic biological agents used in therapy. In this respect, the techniques used in the pharmaceutical industry to optimize the formulation and lyophilization of live viral vaccines might be applied to oncolytic virus preparations.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MG drafted the manuscript, and EEH approved it. GR, RC, and EEH provided conceptual input. All authors participated in the discussion and approved the final submitted version of the manuscript.

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We are grateful for the permission to adapt the figure from Atlas Genet Cytogenet Oncol Haematol, November 2009; Iosco, C; Rhoden, KJ. TSHR (thyroid stimulating hormone receptor). http://atlasgeneticsoncology.org/Genes/TSHRID290ch14q31.html by permission of the Atlas.

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Exploring Leptin Antagonism in Ophthalmic Cell Models

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Abstract

Background: Emerging evidence suggests that angiogenic and pro-inflammatory cytokine leptin might be implicated in ocular neovascularization. However, the potential of inhibiting leptin function in ophthalmic cells has never been explored. Here we assessed mitogenic, angiogenic, and signaling leptin activities in retinal and corneal endothelial cells and examined the capability of a specific leptin receptor (ObR) antagonist, Allo-aca, to inhibit these functions.

Methods and Results: The experiments were carried out in monkey retinal (RF/6A) and bovine corneal (BCE) endothelial cells. Leptin at 50-250 ng/mL stimulated the growth of both cell lines in a dose-dependent manner. The maximal mitogenic response (35±7 and 27±3% in RF6A and BCE cells, respectively) was noted at 24 h of 250 ng/mL leptin treatments. Leptin-dependent proliferation was reduced to base levels with 10 and 100 nM Allo-aca in BCE and RF6A cells, respectively. In both cell lines, leptin promoted angiogenic responses, with the maximal increase in tube formation (163±10 and 133±8% in RF6A and BCE cultures, respectively) observed under a 250 ng/mL leptin treatment for 3 h. Furthermore, in both cell lines 250 ng/mL leptin modulated the activity or expression of several signaling molecules involved in proliferation, inflammatory activity and angiogenesis, such as STAT3, Akt, and ERK1/2, COX2, and NFκB. In both cell lines, leptin-induced angiogenic and signaling responses were significantly inhibited with 100 nM Allo-aca. We also found that leptin increased its own mRNA and protein expression in both cell lines, and this autocrine effect was abolished by 100-250 nM Allo-aca.

Conclusions: Our data provide new insights into the role of leptin in ocular endothelial cells and represent the first original report on targeting ObR in ophthalmic cell models.

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Competing interests: The compound described in the paper, Allo-aca (leptin receptor antagonist peptide) is covered by the 20120142585 patent application (Leptin antagonist and methods of use) filed on Mar 30, 2010, issued on Jun 07, 2012. The patent is assigned to Temple University. Drs. Surmacz and Otvos Jr. as co-inventors might benefit from future patent licensing. This study was supported by a research grant from Novo Nordisk Diabetes Innovation Award. None of the authors is an employee or consultant for this company, and the company does not own or license patents on compounds described in this study. According the Novo Nordisk award agreement, any and all research results and reports, whether patentable or not, are the sole and exclusive intellectual property of Recipient and/or Institution. The company does not influence or prescreen data submitted for publication. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Angiogenesis plays a central role in adult tissue homeostasis and is also responsible for several pathological conditions, including those affecting the eye [1,2]. Ocular neovascularization is a pathological hallmark of some forms of vision-threatening complications, including proliferative diabetic retinopathy (PDR), age related macular degeneration (AMD) and corneal pathologies [2-5].

The complex pathophysiology of ocular neovascularization reflects impairment of metabolic, endocrine and hematologic systems, which leads to the development of local imbalance between pro-angiogenic/inflammatory factors and their modulators [2,4]. The overexpression of vascular endothelial growth factor (VEGF) is thought to be the leading cause of abnormal vessel formation in the eye. However, several other activators of angiogenesis such as platelet-derived growth factor, basic fibroblast growth factor (bFGF), hepatocyte growth factor, interleukins 1a, 6 and 8, and leptin have also been implicated [6]. Many of these factors act through upregulation of VEGF synthesis but their direct involvement remains largely unclear [1,6]. At present, VEGF targeting drugs (i.e., ranibizumab, a modified anti-VEGF antibody and aflibercept, a VEGF trap fusion protein) are approved for the treatment of wet AMD and diabetic macular edema (DME), and experimentally used for other eye diseases, e.g., PDR [7]. However, adverse

effects (systemic and ocular) and development of resistance to the treatment have been noted with long-term use. Thus, targeting pro-angiogenic factors other than VEGF could be prove to be an effective alternative or complementary therapy for pathological neovascularization in the eye [4,6-9].

This study focuses on molecular targeting of pro-angiogenic action of leptin in retinal and corneal cell models. Leptin, a pluripotent cytokine has been first described as an adipocyte-derived hormone that regulates energy expenditure and food intake via hypothalamic effects [10,11]. Later studies proved that leptin is expressed in different peripheral organs and tissues and is involved in multiple physiological and pathological processes, such as immune response, hematopoiesis, fertility, bone remodeling, cardiovascular disease, type 2 diabetes, and cancer [12-16].

Of special interest is the ability of leptin to regulate normal and abnormal angiogenesis. The leptin receptor (ObR) was detected in vascular endothelial cells and studies in vitro demonstrated that leptin can induce angiogenic differentiation, migration and proliferation in endothelial cells. Most of these studies were carried out using human umbilical vein endothelial cells (HUVEC) or aortic endothelial cells [17-23]; only one study involved retinal endothelial cells [24].

Leptin exerts its effects through multiple intracellular signals, including the Janus kinase 2/signal transducer and activator of transcription (JAK2/STAT3), Ras/extracellular signal-regulated kinase 1/2 (Ras/ERK1/2), phosphoinositide 3 kinase/protein kinase B/glycogen synthase kinase 3 (PI-3K/Akt/GSK3) as well as pro-inflammatory cyclooxygenase 2 (COX2) and nuclear factor kappa B (NFκB) pathways [21,25-28]. In HUVEC, the use of specific inhibitors suggested that leptin-mediated angiogenesis depends on ObR crosstalk with VEGFR2 and is mediated through a functional axis involving $p38^{MAPK}$, Akt/PI3K/Akt, and COX2 [21]. Interestingly, some studies show that leptin-induced angiogenesis in HUVEC can be partially reduced with VEGFR inhibitor [21], while others did not observe such effects [29], suggesting independent leptin action.

A few recent studies addressed the role of leptin in ophthalmic experimental models. ObR was detected in primary porcine retinal endothelial cells and leptin treatment stimulated STAT3 phosphorylation and induced VEGF mRNA expression in this model [24]. In a corneal angiogenic assay, leptin stimulated vessel formation synergistically with FGF [23]. However, leptin was not able to induce neovascularization in corneas from fa/fa Zucker rats that lack functional ObR [20]. In mouse models, transgenic overexpression of the leptin gene (*ob*) potentiated ischemia-induced retinal neovascularization, while leptin deficiency due to ob inactivation, significantly reduced ocular angiogenesis. Leptin action in ob transgenic mouse model was mediated, at least in part, through increased VEGF expression [24]. Noteworthy, alkali-induced corneal neovascularization in normal mice was associated with leptin and VEGF overexpression in the regions of new vessels formation [30].

While experimental data suggested leptin involvement in ocular neovascularization, relevant clinical reports are scarce and occasionally conflicting. Gariano et al. demonstrated that in a group of 48 patients with proliferative diabetic retinopathy (PDR) or retinal detachment (RD), intravitreous leptin levels were significantly elevated relative to leptin expression in the eyes of patients with other ocular diseases [31]. In addition, the study suggested that locally produced leptin, not simply leptin derived form circulation, could be involved in the pathogenesis of PDR and RD [31]. Similarly, a small study confirmed higher vitreous leptin levels in PDR relative to other retinopathies [32]. On the other hand, other preliminary analysis involving 25 patients with PDR demonstrated that intravitreous leptin was not directly associated with the disease [33].

Until present, blocking leptin signals in experimental ophthalmic models has not been attempted. We recently generated peptide-based compounds that interfere with leptin/ObR binding and downstream signaling [15,34]. The lead ObR antagonist, Allo-aca, is a 9 residue peptidomimetic that inhibits leptin-induced proliferation and signaling at pM-low nM concentrations in vitro and exhibits anti-neoplastic and anti-inflammatory activities in vivo at 0.1-0.5 mg/kg/day doses [35-37]. The efficacy of Allo-aca in endothelial cells has never been addressed and is explored here in retinal and corneal cell models.

Methods

Reagents

The ObR antagonist, Allo-aca, is a short leptin-based peptidomimetic (H-alloThr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2) whose sequence is based on leptin/ObR binding site III. The design, development and efficacy of Allo-aca in vitro and in vivo have been reported by us before [35,38-40]. An unrelated peptide Chex1-Arg20: H-Chex-Arg-Pro-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Arg-Pro-Val-Arg-NH_2 was used as control [37].

Leptin (human recombinant) and VEGF (human recombinant, VEGF 165) were purchased from R&D Systems (Minneapolis, MN).

Cell lines and growth conditions

Monkey endothelial retinal cells (RF/6A) and bovine endothelial corneal cells (BCE) were purchased from the American Type Culture Collection (Rockville, MD, USA). RF/6A cells were grown in Minimum Essential Medium (MEM) containing 1 g/L glucose, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). BCE cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% FBS and 1% P/S. All culture reagents and media were purchased from Cellgro (Cellgro, Herndon, VA, USA).

Proliferation assay

The cells (2-5^{th} passage) were plated in 24 well plates at concentrations 8 $x10^4$ and $1x10^5$ cells/well for RF/6A and BCE cells, respectively. At 70% confluence, the cells were shifted to serum-free medium (SFM: MEM or DMEM with 10 μM $FeSO_4$, 0.5% bovine serum albumin, 1% FBS, 1% P/S) for 24 h and then treated with 50-500 ng/mL of leptin (R&D) for 24 or 48 h.

The effects of 10-500 nM Allo-aca on leptin mitogenic activity were tested following the analogous protocol. Cell numbers before and after treatments were determined by direct counting with trypan blue exclusion. All assays were done in triplicate and repeated 3-6 times. The percentage decrease/increase in cell number vs. control SFM was calculated and expressed as mean ± standard deviation (SD).

Intracellular signaling

To assess short-term effects of leptin in RF/6A and BCE cells, 70% confluent cell cultures at 4th or 5th passage were shifted to SFM for 24 h and then treated with 250 ng/mL leptin for 15 or 30 min, or left untreated. To test the effects of Allo-aca on leptin signaling, the cells were pretreated with the antagonist at 10-100 nM for 1 h before leptin addition. The long-term effects of leptin and Allo-aca were determined at 6, 12, and 24 h post treatment. Next, the cells were lysed and total cellular proteins were obtained as described previously [41]. The expression of ObR and downstream signaling molecules was evaluated by Western Blot (WB) in 50-100 µg of total proteins. The following primary antibodies (Abs) from Cell Signaling Technology (Danvers, MA) were used: for phospho-Akt, Akt Ser473 pAb, 1:500; for total Akt, Akt pAb, 1:1000; for phospho-STAT3, STAT3 Tyr705, D3A7 mAb, 1:500; for total STAT3, STAT3 79D7 mAb, 1:500; for phospho-ERK1/2, p44/42 mitogen-activated protein kinase (MAPK; ERK1/2) pAb Thr202/ Tyr204, 1:1000; for total ERK1/2, p44/42 MAPK pAb, 1:1000; for total COX2, COX2 pAb, 1:250. The following primary Abs from Santa Cruz were used: for ObR, H-300 pAb, 1:500; for NFκB, for NFκB p65A, pAb 1:500; for β-actin, Actin I-19 pAb, 1:500.The intensity of bands corresponding to studied proteins was measured using ImageJ program as described before [22].

Angiogenic assay

The ability of cells to migrate and organize into enclosed spaces (ES) on Matrigel was carried out as described in detail previously [22]. Briefly, the cells at 4th or 5th passage were shifted to SFM for 24 h. Next, the cells at 1x10^4 (RF/6A) and 2x10^4 (BCE) were suspended in 200 µl SFM containing either leptin at different concentrations, leptin plus Allo-aca at different concentrations, Allo-aca alone, control unrelated peptide, or VEGF at different concentrations. SFM alone was used as a negative control. The mixtures were seeded in 96 well plates covered with polymerized growth factor-reduced Matrigel matrix (BD, Franklin Lakes, NJ), incubated for 3 h at 37°C and photographed using Olympus 1x81 phase-contrast microscope at 3.2 x magnification and Metamorph 7.5 program. The number of ES in the whole photographed area (representing central 70% of the well) was scored by two observers. Each experiment was done in triplicate and repeated at least 3 times. The mean number of ES ± SD was determined for each condition.

Quantitative Real Time PCR (qRT-PCR)

RF/6A and BCE cells at 70% confluence were shifted to SFM for 24 h and then treated with 250 ng/mL leptin for 3, 6, 24 h. To test the effects of Allo-aca, the cultures were pretreated 10-250 nM Allo-aca for 1 h. RNA was isolated using Trizol Reagent (Life Technologies, Grand Island, NY) according to manufacturer's instructions. A total of 4 µg of RNA was reverse transcribed in 20 µL of reaction volume using the High-Capacity cDNA Kit (Life Technologies). Four µL of the RT product were used to amplify leptin sequences using TaqMan probes Bt03211909_m1 for bovine leptin and Rh02788316_m1 for monkey leptin (Life Technologies). To normalize qRT-PCR results, parallel reactions were run on each sample for β-actin using a TaqMan probe (Life Technologies). The levels of target mRNA relative to β-actin mRNA were determined using a comparative CT method, as suggested by the manufacturer (Life Technologies). All reactions were done in triplicate and an average CT value (±SD) for all RNAs was calculated. The individual experiments were repeated at least 3 times.

Immunofluorescence

Leptin protein was detected in RF/6A and BCE cells by immunofluorescence (IF), as described by us before [42]. In short, 1x10^5 cells were plated on sterile glass cover slips in normal growth medium. After 24 h, the cells were synchronized in SFM for 24 h and then treated with 250 ng/mL leptin in the presence or absence of 100 or 250 nM Allo-aca for 24 h. Next, the cells were washed with PBS, fixed for 10 minutes in methanol at -20°C, and permeabilized in 0.2 Triton X-100% for 5 min at room temperature. Leptin expression was detected using pAb A-20 (1:25 dilution; 2 h) and goat anti-rabbit IgG-FITC (1:1000 plus 1.5% blocking goat serum; 1 h). In control experiments, primary Abs were replaced by non-immune serum. Following staining, the coverslips were mounted using UltraCruz Mounting Medium containing 1.5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) to allow visualization of cell nuclei. All Abs and other reagents were purchased from Santa Cruz Biotechnology. The expression of leptin under different conditions was assessed using Olympus 1x81 phase-contrast microscope at 2 x magnification and Metamorph 7.5 program. The percentage of positive cells was determined in 10 visual fields.

Statistical analysis

All experiments were done at least in triplicates and data analyzed by Student's t-test.

Differences with p values of ≤ 0.05 were considered significant.

Results

ObR antagonist, Allo-aca, inhibits leptin growth effects in RF/6A and BCE cells

We first tested leptin time and dose-responses in RF/6A retinal and BCE corneal cells. Leptin was used at 50-500 ng/mL concentrations and cells were treated for 24-72 h. The maximal growth responses in both cell lines were observed at 24 h stimulation (data not shown), thus this time point was used in further experiments. In RF/6A and BCE cells, a 24 h leptin treatment induced cell growth in a dose-dependent manner at 50-250 ng/mL. The maximal mitogenic effect, i.e., ~35% and 27%, in RF6A and BCE cells, respectively, was

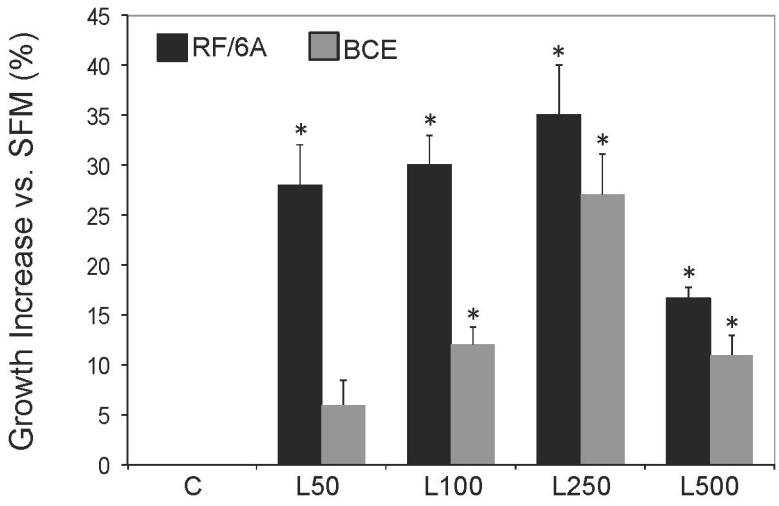

Figure 1. Leptin growth response in RF/6A retinal and BCE bovine corneal endothelial cells. RF/6A and BCE cells were synchronized in SFM and stimulated with 50-250 ng/mL leptin (L) for 24 h. The % increase (±SD) in cell number vs. untreated control (C=SFM) is shown. Asterisks indicate significant (p≤0.05) differences vs. SFM.
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observed with 250 ng/mL, while the response declined under 500 ng/mL leptin concentrations (Figure 1).

In both cell lines, the treatment with 10-250 nM Allo-aca significantly reduced mitogenic activity of 250 ng/mL leptin. A complete inhibition was observed with 100 nM Allo-aca concentrations, while at 250 nM, the peptide reduced cell proliferation ~20% below base levels (Figure 2), possibly by inhibiting endogenous leptin expression (see below). When added alone, Allo-aca did not produce any significant cytostatic or cytotoxic effects at 1-100 nM concentrations. In RF/6A cells, a weak (9%) agonist activity of 250 nM Allo-aca was detected. This effect, however, was not noted in BCE cells. A control peptide was inactive in this assay (data not shown).

ObR antagonist blocks leptin-induced angiogenic effects in RF/6A and BCE cells

In both cell lines, leptin increased ES formation at 50-250 ng/mL concentrations. The maximal stimulation of tube formation (by ~3.0- and 2.75-fold in RF/6A and BCE cells, respectively) was noted under the 250 ng/mL leptin treatment for 3 h (Figure 3). This effect was comparable to that of 100 ng/mL VEGF in RF6A and 200 ng/mL VEGF in BCE cells (Figure 3 and data not shown).

The angiogenic activity of 250 ng/mL leptin was totally blocked by Allo-aca at 50-100 nM concentrations in both cell lines (Figure 4). Allo-aca alone did not affect tube formation up to 100 nM concentrations, and a control peptide was totally neutral in angiogenesis assays (Figure 4 and data not shown).

ObR antagonist inhibits several leptin-dependent acute and long-term intracellular responses in RF/6A and BCE cells

Leptin at 250 ng/mL activated STAT3, ERK1/2, and Akt in both cell lines at 15 min. Specifically, the phosphorylation of STAT3 was upregulated by 64 and 58%, of ERK1/2 by 65 and 49%, and of Akt by 21 and 55% in RF/6A and BCE, respectively. The response to leptin was in most cases less pronounced at 30 min (Figure 5). The short leptin exposure did not affect the expression of p65 NFκB and COX2 (the latter was barely detectable in BCE cells) (Figure 5).

We also studied long-term effects of leptin stimulation on the expression and/or activation of major ObR downstream targets (Figure 6). In general, the maximal responses were noted at 6-12 h in RF/6A cells and at 6 h in BCE cells. In RF/6A cells, a prolonged exposure to 250 ng/mL leptin increased the expression of COX2 by ~38%, decreased the expression of NFκB by 16% as well as upregulated STAT3, ERK1/2 phosphorylation by 18% and 48%, respectively. In BCE cells, 6 h leptin stimulation did not modulate COX2 or NFκB, but increased STAT3 and Akt phosphorylation by 48 and 40%, respectively (Figure 6). Interestingly, 24 h leptin treatment decreased COX2 and NFκB expression in RF/6A cells by 20% and 30%, respectively. The above intracellular responses were significantly reduced in the presence of 100 nM Allo-aca (Figure 7). None of the stimulatory or inhibitory treatments altered the expression of ObR in studied cells (Figures 5-7).

Leptin upregulates leptin mRNA and protein expression in RF/6A and BCE cells. Allo-aca antagonizes this effect.

We found that under basal growth conditions RF6A and BCE cells synthesize leptin mRNA, the latter expressing ~7-fold more than the former (data not shown). The treatment with exogenous leptin for 3, 6, and 24 h further potentiated leptin mRNA expression in both cell lines. The maximal effects of leptin on its own mRNA synthesis, i.e., ~3.6-fold increase in RF/6A at 6-24 h and 1.7-fold increase in BCE cells at 6 h of stimulation (Figure 8A). This autocrine leptin synthesis was abolished by 100-250 nM Allo-aca in RF/6A cells and reduced

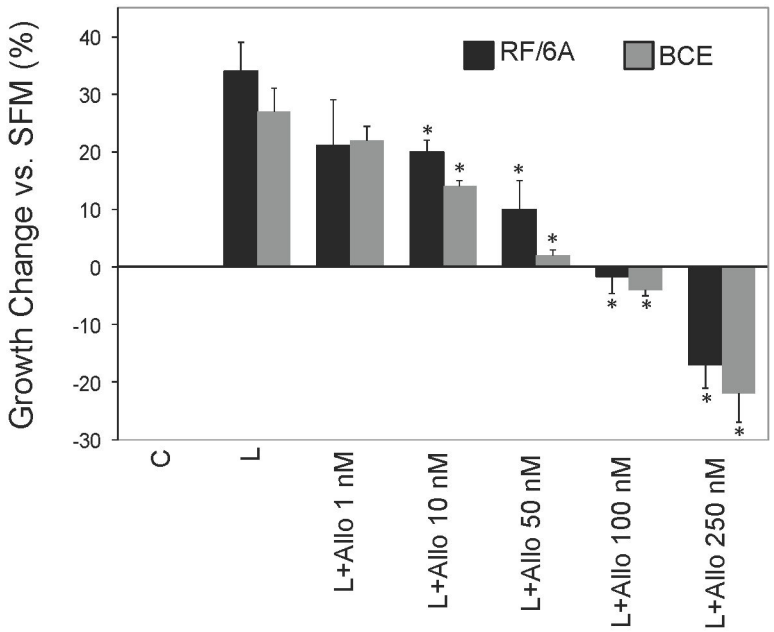

Figure 2. Effects of Allo-aca on leptin-dependent proliferation in RF/6A and BCE cells. RF/6A and BCE cells were synchronized in SFM and stimulated with 250 ng/mL leptin (L) in the presence or absence of 1-250 nM Allo-aca (Allo) for 24 h. The % increase/decrease (±SD) in cell number vs. untreated control (C=SFM) is shown. Asterisks indicate significant ($p \leq 0.05$) differences vs. leptin.

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by ~33% in the presence of 250 nM in BCE cells (Figure 8B). Similarly, we found that both cell lines express endogenous leptin protein. In BCE cultures, ~35% of cells expressed leptin under SFM conditions, while in RF/6A cultures, only ~ 6% of cells were strongly positive for leptin immunostaining. Treatment with leptin increased the number of leptin expressing cells by 60% in RF/6A cultures and by 30% in BCE cells. The increased leptin expression was blocked with 100 and 250 nM Allo-aca in RF/6A and BCE cells, respectively (Figure 9).

Discussion

Ocular neoangiogenesis is one of the mechanisms implicated in vision threatening diseases, such as PDR, AMD and some corneal pathologies [2]. Current treatments for these diseases include laser photocoagulation, vitrectomy surgery and/or intravitreal injections of anti-VEGF agents. At present, FDA-approved treatments for wet AMD and DME include modified anti-VEGF antibodies and VEGF trap proteins. These drugs are experimentally used for other eye diseases, e.g., PDR [7].

In addition to VEGF and VEGFR, other mediators of ocular neovascularization are being considered as potential therapeutic targets [4]. This includes leptin, an upstream activator of VEGF pathways as well as an independent angiogenic and inflammatory agent [6,21].

Although leptin is known to promote angiogenesis and endothelial cell growth [18,21,22,43], its function in ophthalmic cell models has not been systematically explored. Only one study described leptin effects in porcine retinal endothelial cells and provided a very limited data on ObR signaling [24]. Similarly, targeting ObR in ophthalmic in vitro or in vivo models has never been attempted.

Here, we studied biological effects of leptin and consequences of interfering with leptin signals in RF/6A retinal and BCE corneal cells that are accepted preclinical ophthalmic in vitro models [44-50]. In both cell lines, leptin stimulated mitogenesis at 24-72 h at 50-250 ng/mL concentrations. The observed ~30-35% growth response in response to 50-250 ng/mL leptin treatment in RF/6A and BCE cells is consistent with data obtained in non-ophthalmic endothelial cells [18,19,21-23,51] and other cell types [41,52]. Notably, the mitogenic concentrations of leptin in RF/6A and BCE cells were in the upper range of vitreous leptin levels reported in PDR patients (~37 ng/mL) [32].

In this report, we demonstrated that biological leptin effects were paralleled by the activation of several acute and long-term responses. Intracellular leptin signaling in RF/6A and BCE cells was in part similar to that described for other cell types [14,21,27,34,41,53] but also exhibited some unique features. In both cell lines, short-term leptin exposure activated the STAT3, Akt, and ERK1/2 pathways but did not modulate the expression of the inflammatory mediators COX2 and p65 NFκB. In contrast, more variability was noted under long-term leptin exposure. In both cell lines, 6 or 12 h leptin stimulation increased STAT3 phosphorylation. The treatment also augmented ERK1/2 activation at 6-12 h in RF/6A, but not in

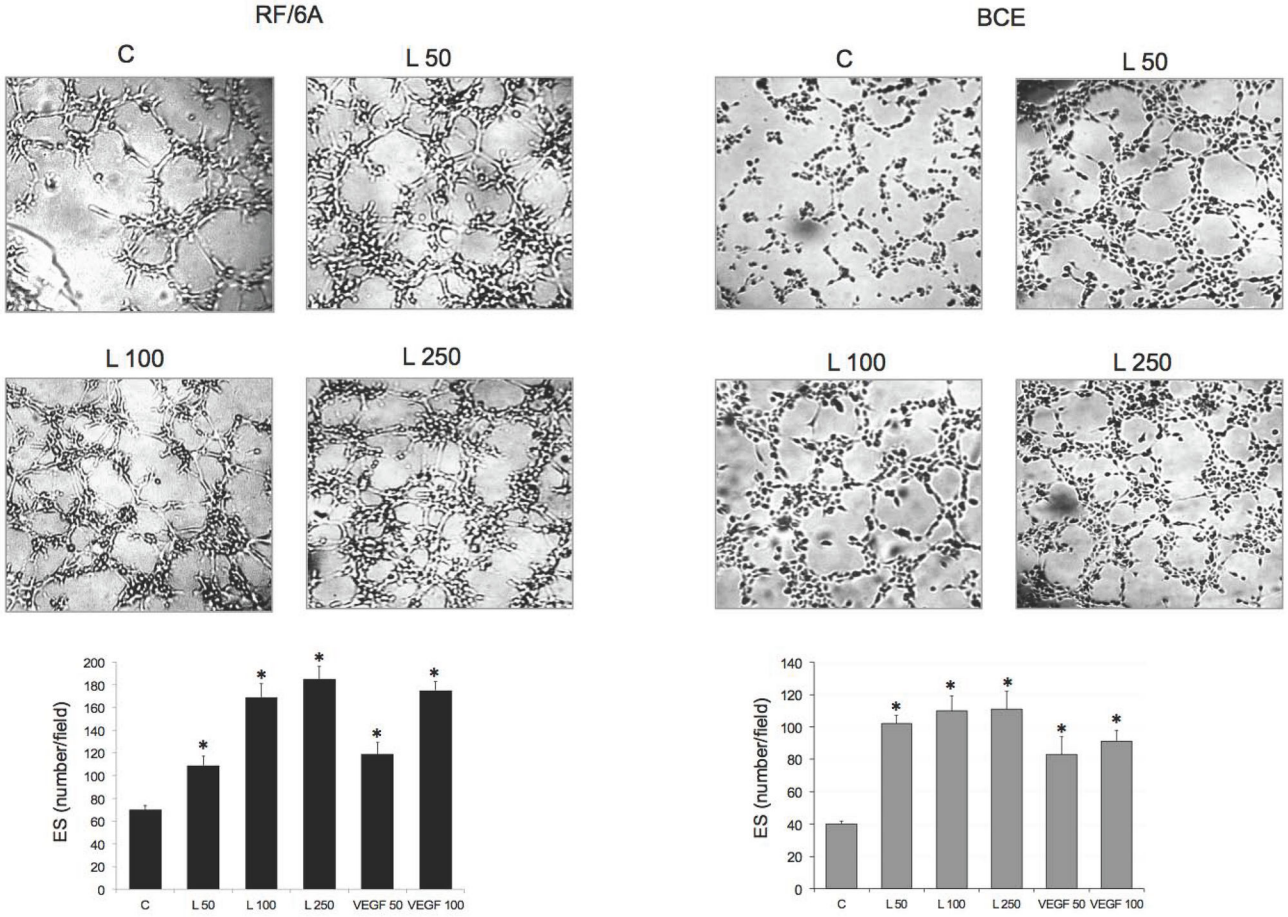

Figure 3. Effects of leptin on angiogenic response in RF/6A and BCE cells. The assays were carried out as described in Materials and Methods. The photographs represent ES formation in RF/6A and BCE cultures under different treatments: SFM (C), 50-250 ng/mL leptin (L), 50 and 100 ng/mL VEGF (central field of the well at 5x magnification is shown). The graph shows the number of ES (±SD) per visual field. Asterisks indicate significant ($p \leq 0.05$) differences vs. SFM.

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BCE cells. On the other hand, the activation of Akt was seen only in BCE cells at 6 h. The reason for these variations in signaling dynamics is unknown. It might reflect the levels of signaling molecules as well as dynamics in endogenous leptin synthesis.

Garonna et al. described that prolonged leptin exposure upregulated COX2 expression in HUVEC [21]. Similarly, we noted significantly increased COX2 expression at 6 and 12 h in RF/6A cells, which was followed by a decrease in COX2 content at 24 h. However, in BCE cells, expressing very low COX2 levels, leptin did not affect this protein in a significant manner. We also noted cell-specific leptin effects on the NFκB pathway. Specifically, we and others described in various non-ophthalmic models that leptin can stimulate NFκB through upregulation of p65 (Rel A) phosphorylation [37,53-55]. In this study, however, we found a progressive decrease of p65 NFκB levels at 6-12 h of leptin exposure in RF/6A cells. This was somewhat surprising as coordinate expression of NFκB and COX2 was described in RF/6A cells in response to hypoxic conditions, and NFκB activation appeared to precede COX2 expression [56]. Potentially, temporal p 65 NFκB downregulation could represent natural oscillation of this factor [57]. One study described activation of p38 kinase in response to leptin treatment in HUVEC [21], however, p38 levels were very low in both our cell lines and we were not able to detect any regulation of this kinase upon leptin short- or long-term leptin treatment (data not shown).

Interestingly, we found that the presence of leptin augments its own mRNA and protein synthesis in both cell lines. While leptin protein was found in ocular tissues by immunohistochemistry and ELISA [31-33,58], we are the first to report that retinal and corneal endothelial cells can regulate leptin expression through an autocrine mechanism. The possibility of intraocular leptin synthesis is also suggested by our preliminary findings that leptin mRNA is expressed in the eyes of animals with laser-induced neovascularization (data not shown). This suggests that retinal and corneal endothelial cells can produce endogenous leptin as well as respond to leptin in

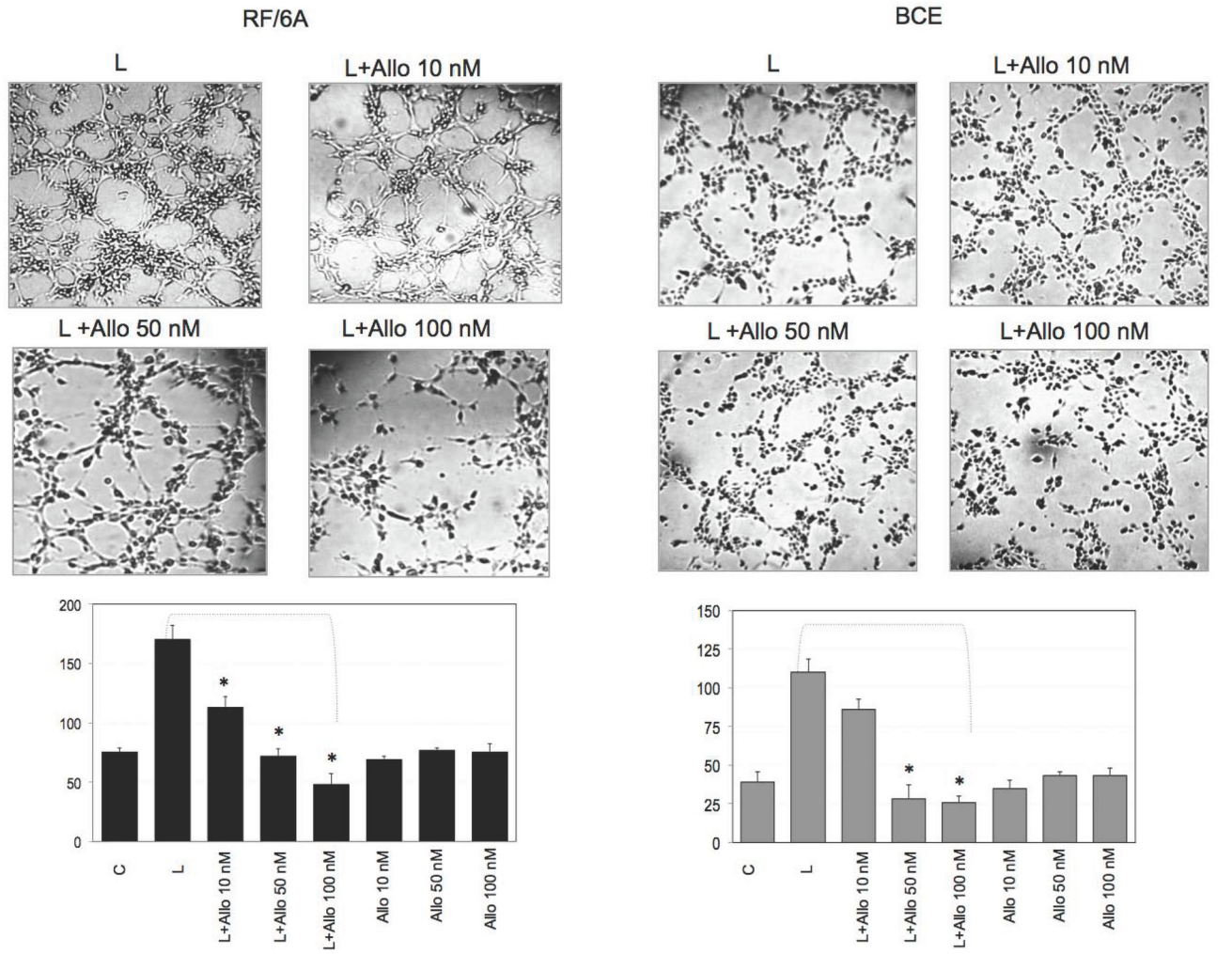

Figure 4. Effects of Allo-aca on leptin angiogenic activity in RF/6A and BCE cells. The assays were carried out as described in Materials and Methods. Untreated cells (C=SFM) and cells treated with 250 leptin (L) alone, L+ 1-100 nM Allo-aca (Allo), or 10-100 nM Allo-aca. The photographs represent ES formation under different treatments in RF/6A and BCE cells (central field of the well at 5x magnification is shown). The graph shows the number of ES (±SD) per visual field. Asterisks indicate significant ($p \leq 0.05$) differences vs. leptin.

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the circulation. Indeed, local and systemic leptin sources in ocular disease were previously suggested based on data obtained in patients [31,32]. In this context, hyperleptinemia associated with diabetes or obesity might influence ocular neovascularization in the situations of compromised blood-retinal barrier.

All of the biological effects of leptin in RF/6A and BCE cells were inhibited by a specific ObR antagonist, Allo-aca. The efficacy of Allo-aca in endothelial cell models has never been tested before and our present data represent the first original report on targeting ObR in ophthalmic cells. In particular, Allo-aca reduced RF/6A and BCE cell growth to basal levels at 100 nM, tube formation at 50-100 nM, cell signaling at 100 nM, and leptin mRNA and protein expression at 100-250 nM concentrations.

In summary, our report provides the original evidence that targeting ObR can reduce leptin mitogenesis and angiogenic differentiation in two different ophthalmic cell lines. Taking into consideration crosstalk between leptin and VEGF systems, one could envision that treatments employing combinations of drugs targeting both pathways could offer better efficacy and limit drug side effects and development of resistance.

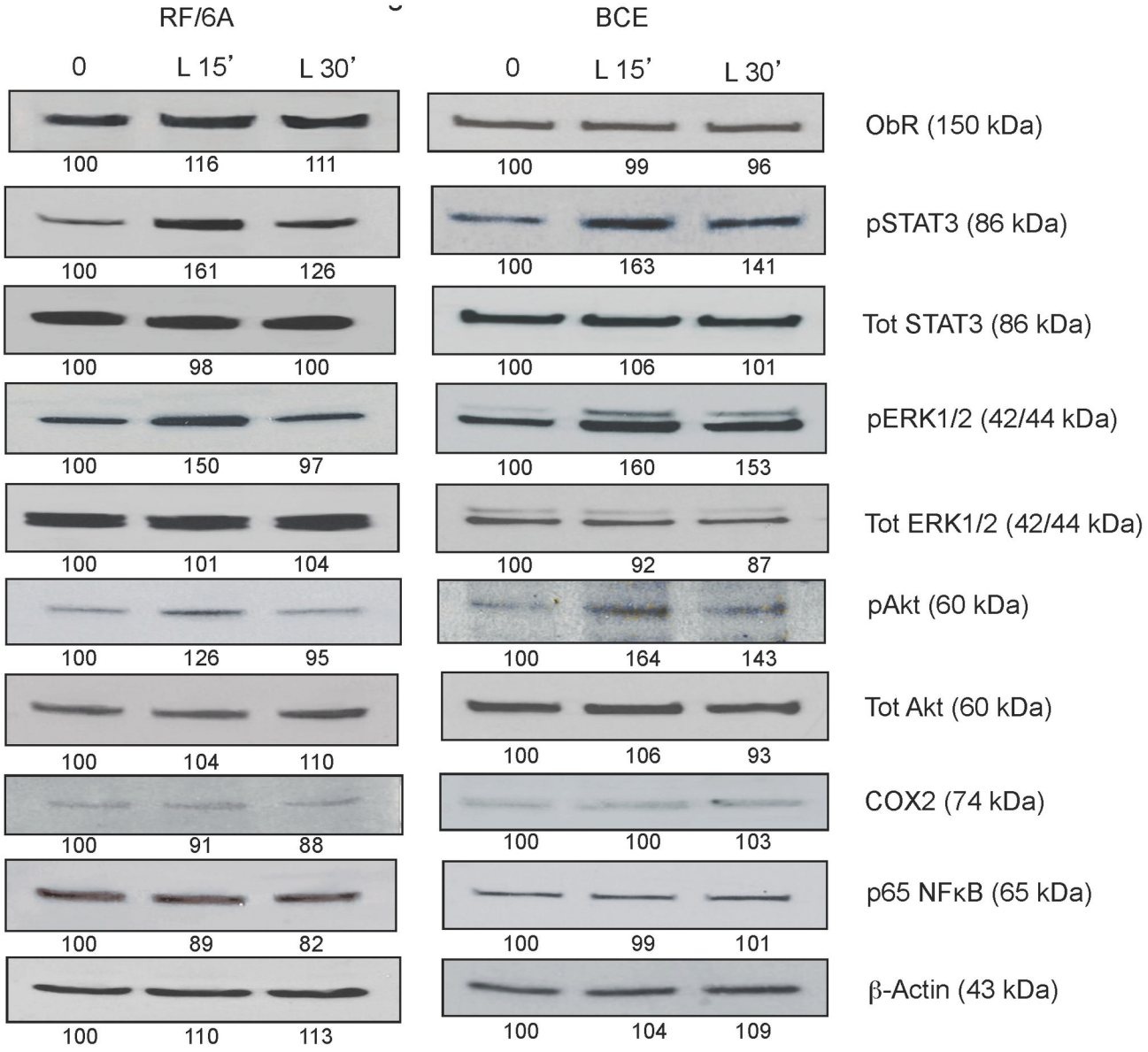

Figure 5. Leptin effects on intracellular signaling in RF/6A and BCE cells. RF/6A and BCE cells were stimulated with 250 ng/mL leptin (L) for 15 and 30 min and the expression of phosphorylated (p) and total (Tot) proteins was assessed by WB and quantified as described in Materials and Methods. The levels of β-actin were assessed as control of loading. The numbers under WB panels represent relative densitometry values (%) of phosphorylated and total proteins, with the value in SFM is taken as 100%. The representative blots of at least 3 experiments are shown.

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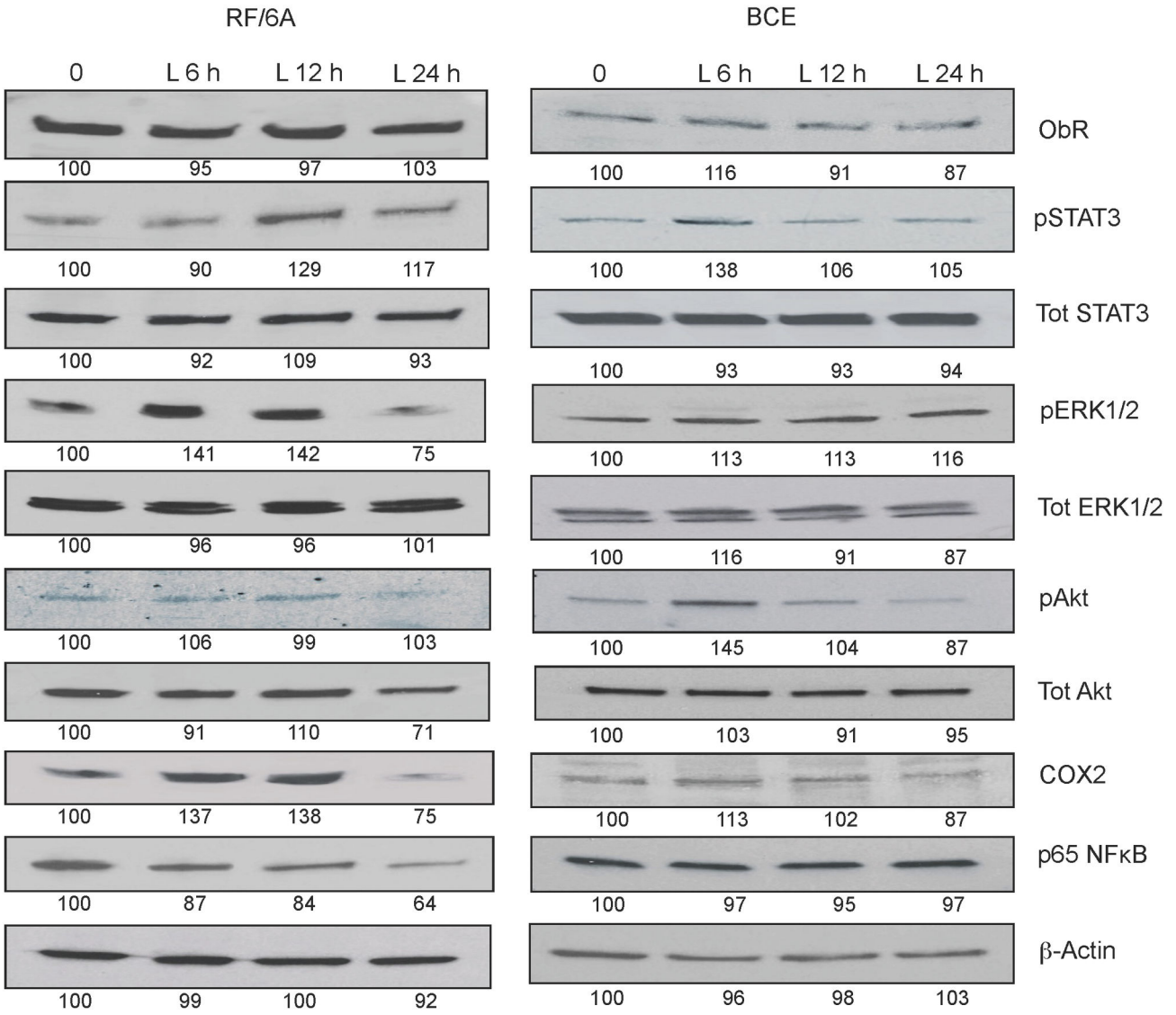

Figure 6. Long-term intracellular leptin effects in RF/6A and BCE cells. RF/6A and BCE cells were stimulated with 250 ng/mL leptin (L) for 6, 12, 24 h as described in Materials and Methods. The numbers under WB panels represent relative densitometry values (%) of phosphorylated and total proteins, with the value in SFM is taken as 100%. The representative blots of at least 3 experiments are shown.

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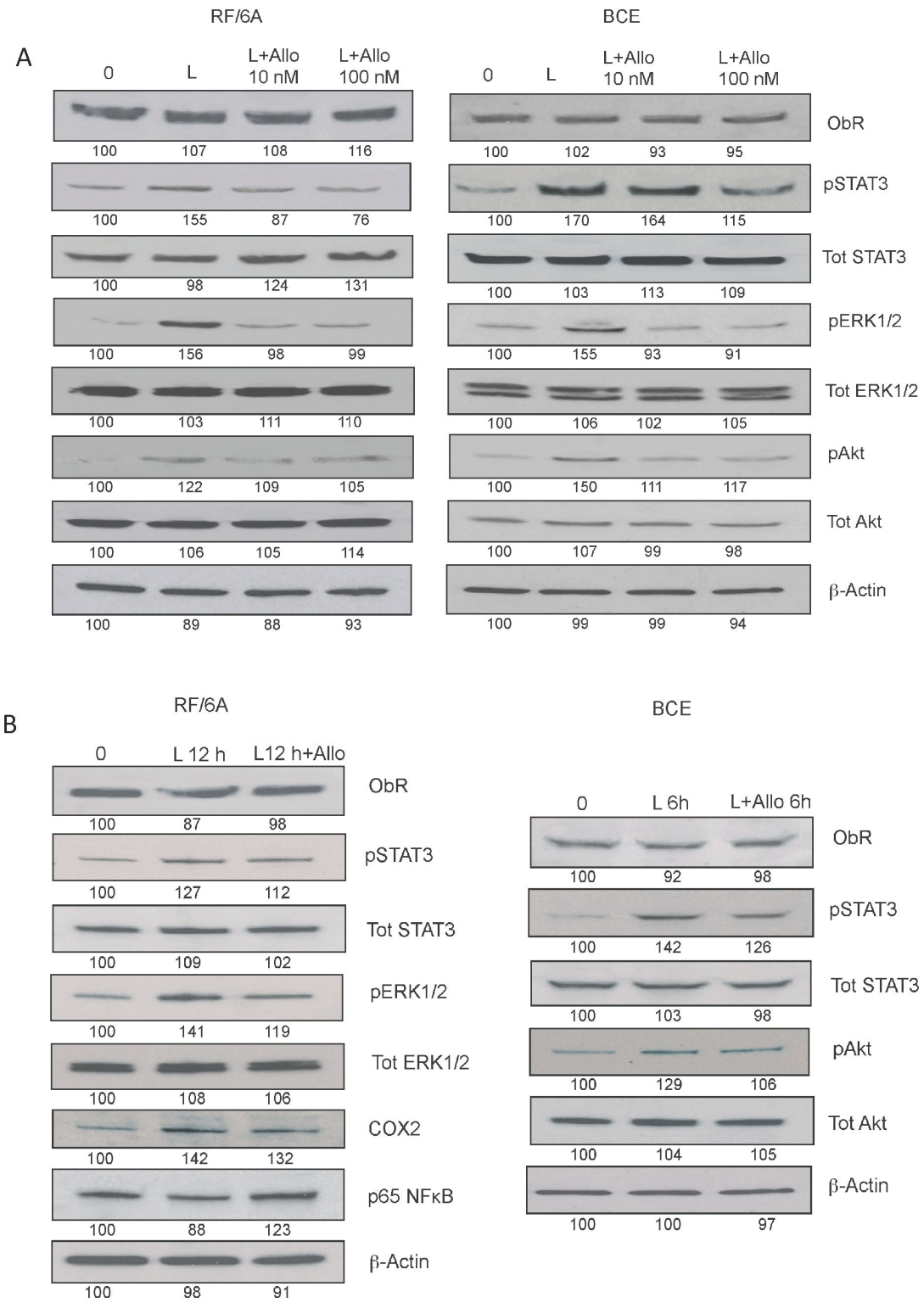

Figure 7. Effects of Allo-aca on intracellular leptin effects in RF/6A and BCE cells. **A**. The effects of 10 and 100 nM Allo-aca (Allo) on acute intracellular signaling were tested in RF/6A and BCE cells stimulated with 250 ng/mL leptin (L) for 30 min. **B**. The long-term effects of 100 nM Allo-aca (Allo) on intracellular pathways were assessed in cells stimulated for 12 h (RF/6A) or 6 h (BCE) with 250 ng/mL leptin (L) and measured as described under Figures 5 and 6. The representative blots of at least 3 experiments are shown.

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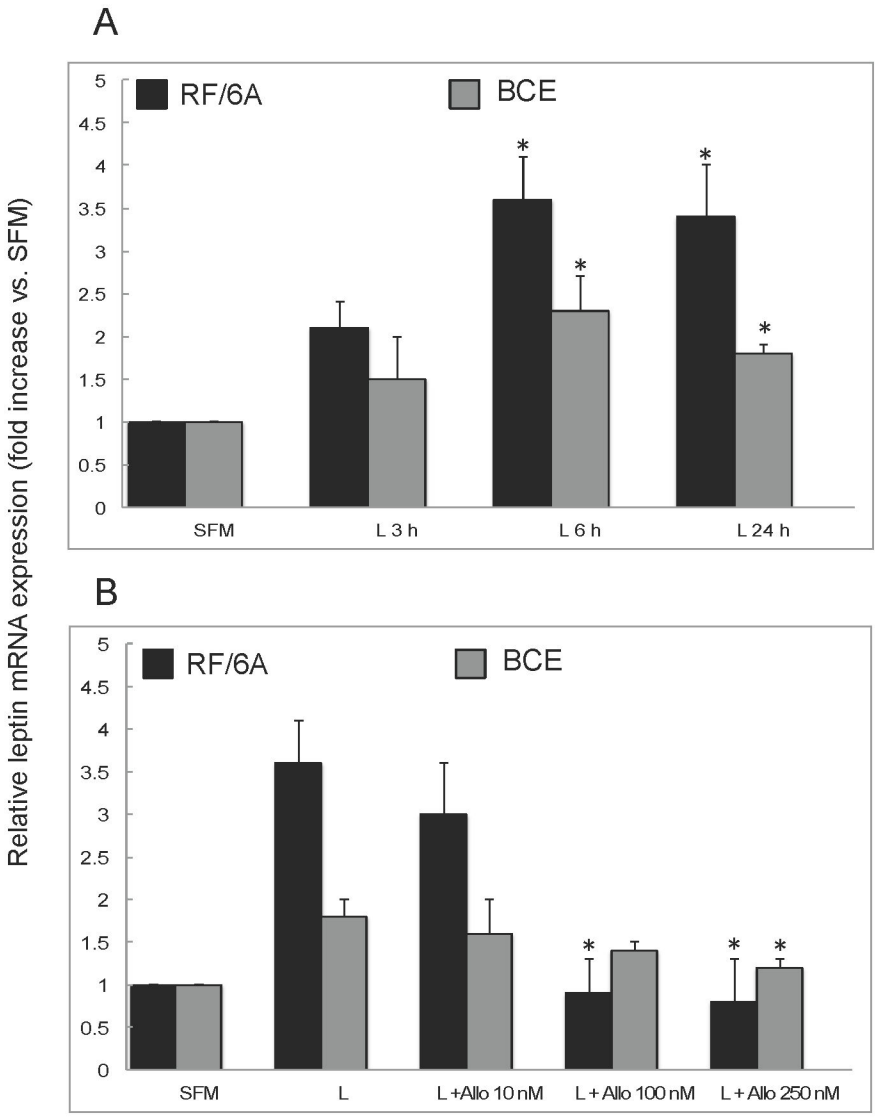

Figure 8. Effects of leptin and Allo-aca on leptin mRNA expression in RF/6A and BCE cells. **A**. RF/6A and BCE cells were stimulated with 250 ng/mL leptin (L) for 3, 6, and 24 h. The expression of leptin mRNA was assessed by QRT-PCR as described in Materials and Methods. The values represent fold increase (± SD) of leptin mRNA levels in leptin treated cells vs. untreated controls (C=SFM) assigned value 1. **B**. The cells were pretreated with 10, 100, or 250 nM Allo-aca (Allo) for 1 h and then stimulated with 250 ng/mL leptin (L) for 6 h. Leptin mRNA was measured by QRT-PCR. Asterisks indicate significant ($p \leq 0.05$) differences vs. SFM (**A**) or leptin (**B**).

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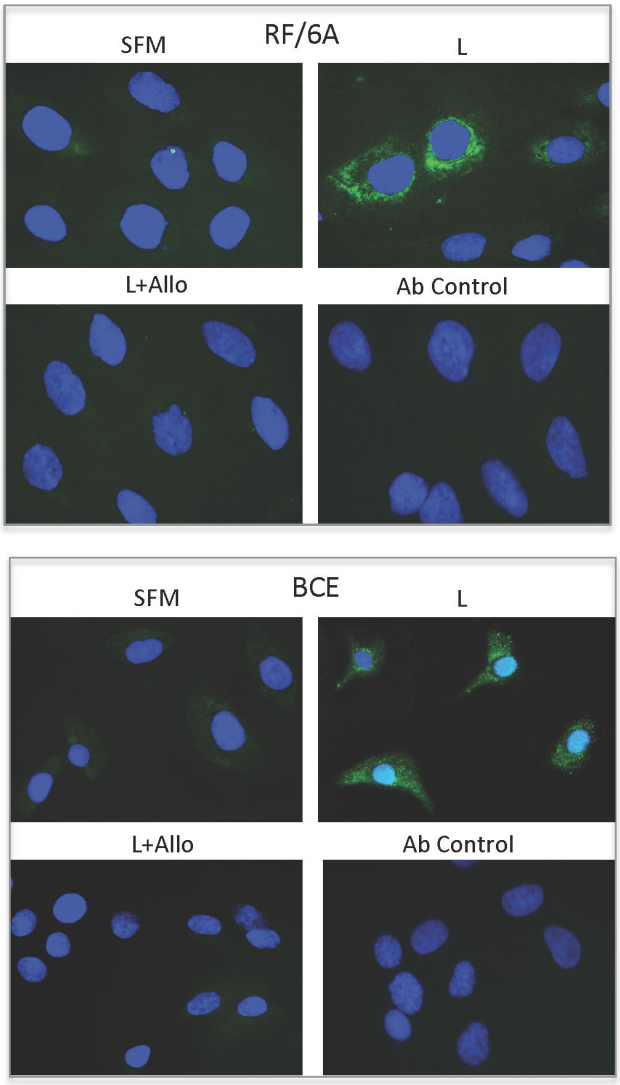

Figure 9. Effects of leptin and Allo-aca on leptin protein expression in RF/6A and BCE cells. RF/6A and BCE cells were synchronized in SFM and stimulated with 250 ng/mL leptin (L) for 24 h in the presence or absence of 100 nM (for RF/6A) or 250 nM (for BCE) Allo-aca (Allo). The expression of leptin protein (green immunofluorescence) in treated and untreated cells was detected with specific Abs, as described in Materials and Methods. In control experiments, the primary Ab was omitted.

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Author Contributions

Conceived and designed the experiments: LS CP ES. Performed the experiments: LS CP RC. Analyzed the data: LS CP RC LO ES. Contributed reagents/materials/analysis tools: LO. Wrote the manuscript: CP ES LS.

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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

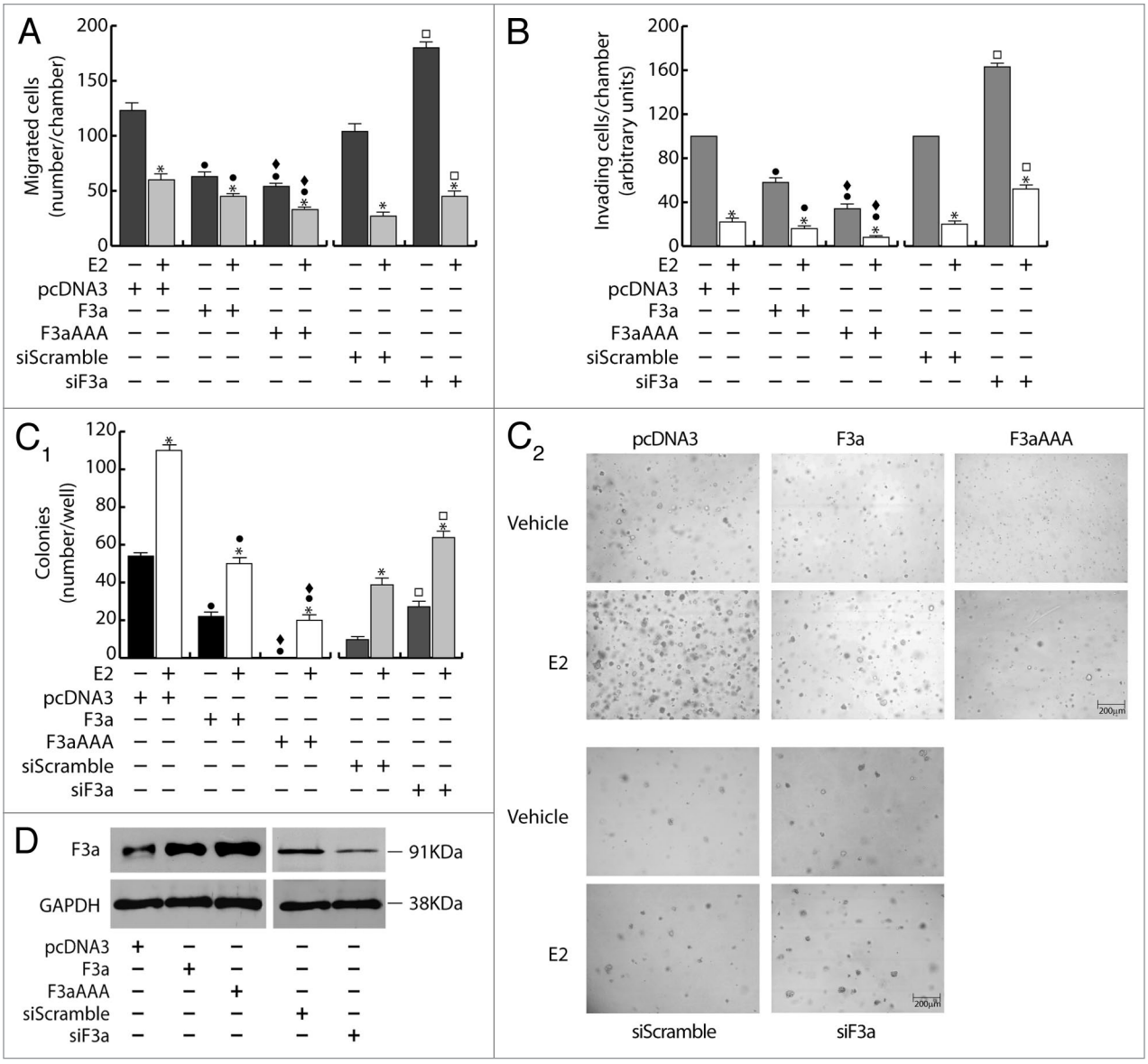


Figure 1. FoxO3a inhibits migration, invasion and anchorage independent growth in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells was transiently transfected with 1 μg/35 mm dish of F3a, F3aAAA, or pcDNA3 as control. Another double set was silenced for FoxO3a expression (siF3a), using a siScramble 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

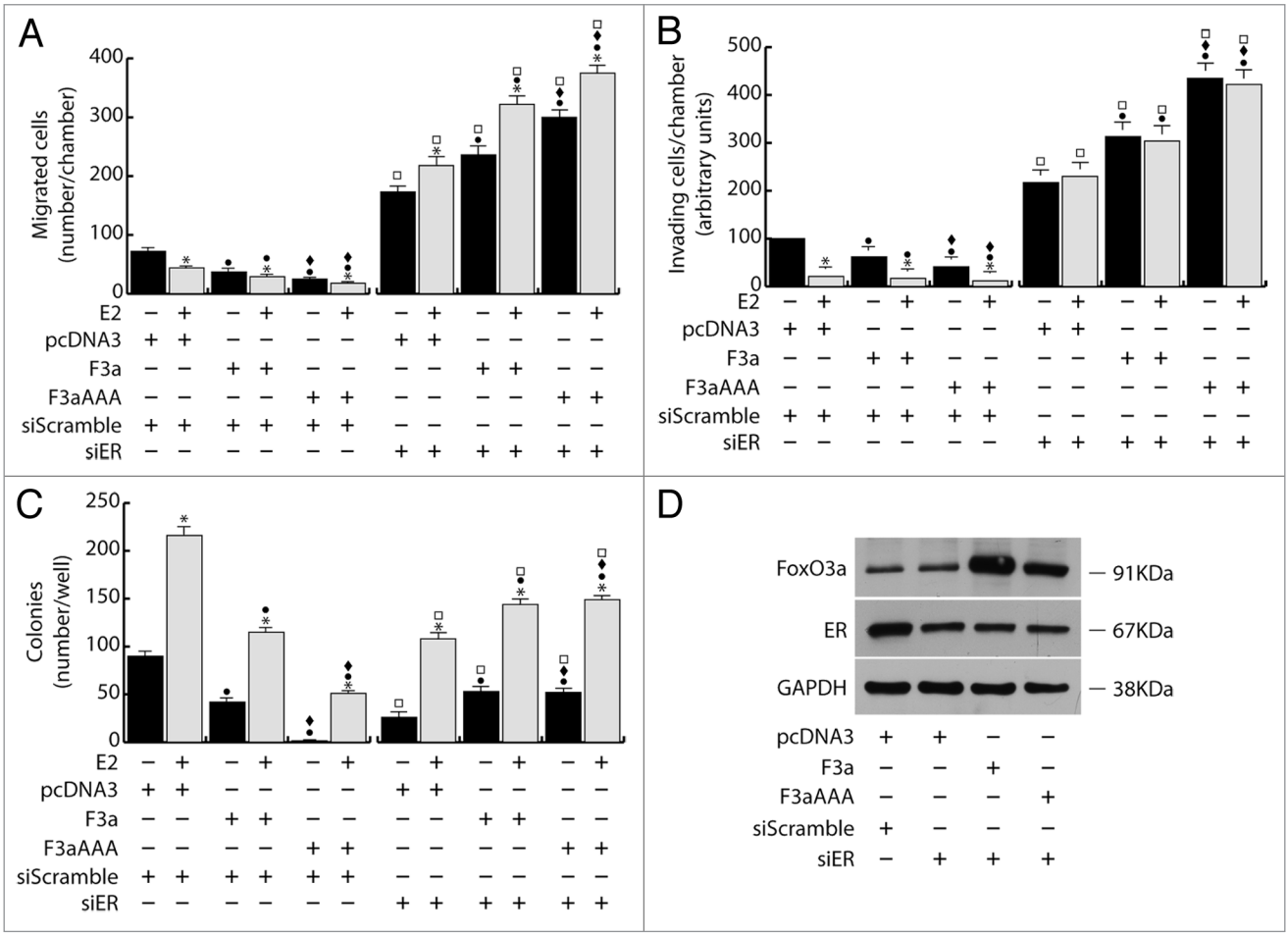


Figure 2. FoxO3a mediated inhibition of breast cancer cell migration, invasion and growth in suspension depends on ERα Two double sets of MCF-7 cells were silenced either for ERα (siER), using siScramble as control. After 5 h cells were switched to PRF-SFM and transiently transfected with F3a, F3aAAA, 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

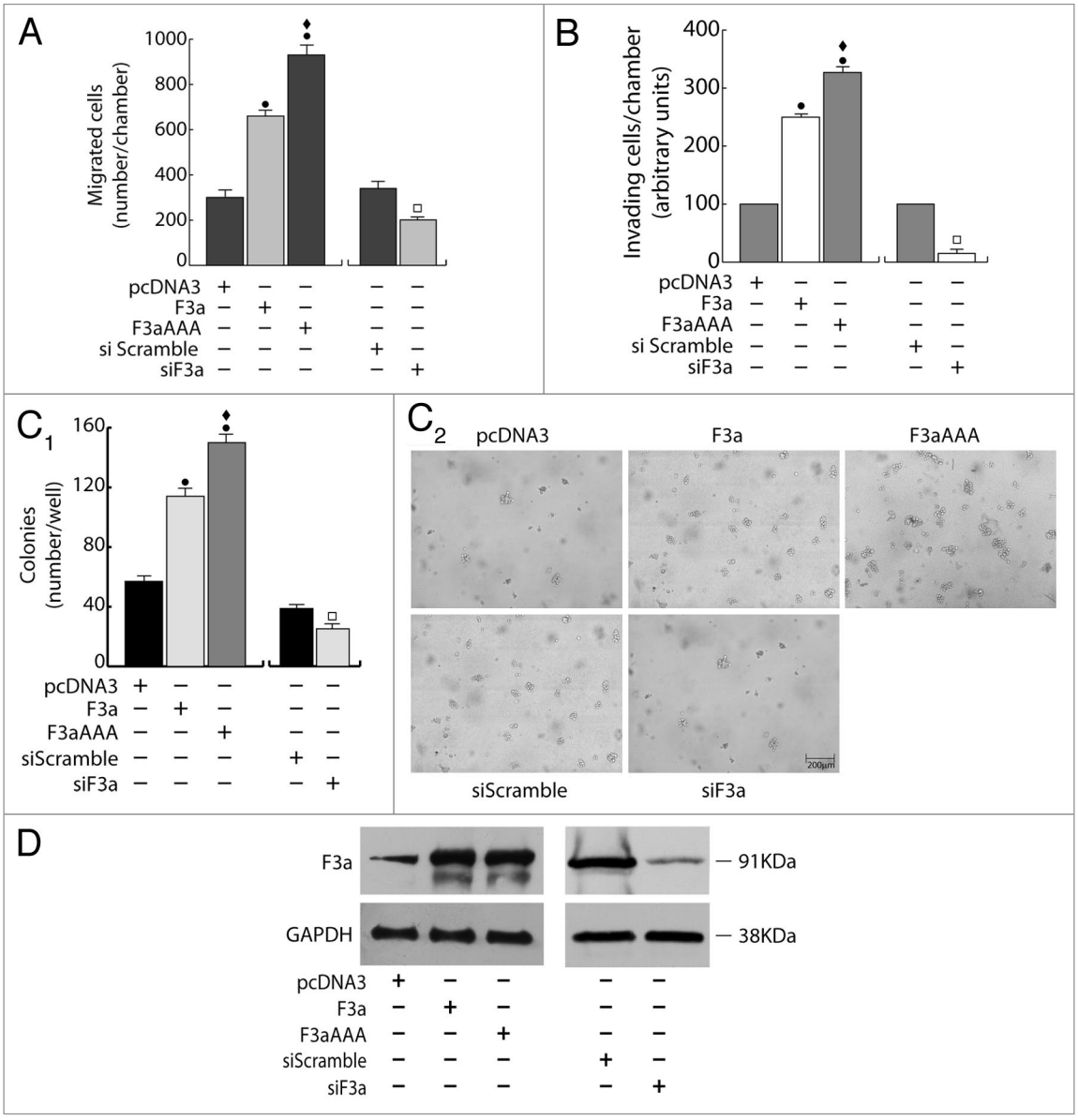


Figure 3. FoxO3a promotes migration, invasion, and anchorage-independent growth in ERα– MDA-MB-231 breast cancer cells. A double set of MDA-MB-231 cells were transiently transfected with 1 μg/35 mm dish of F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a expression (siF3a) using a siScramble as control (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

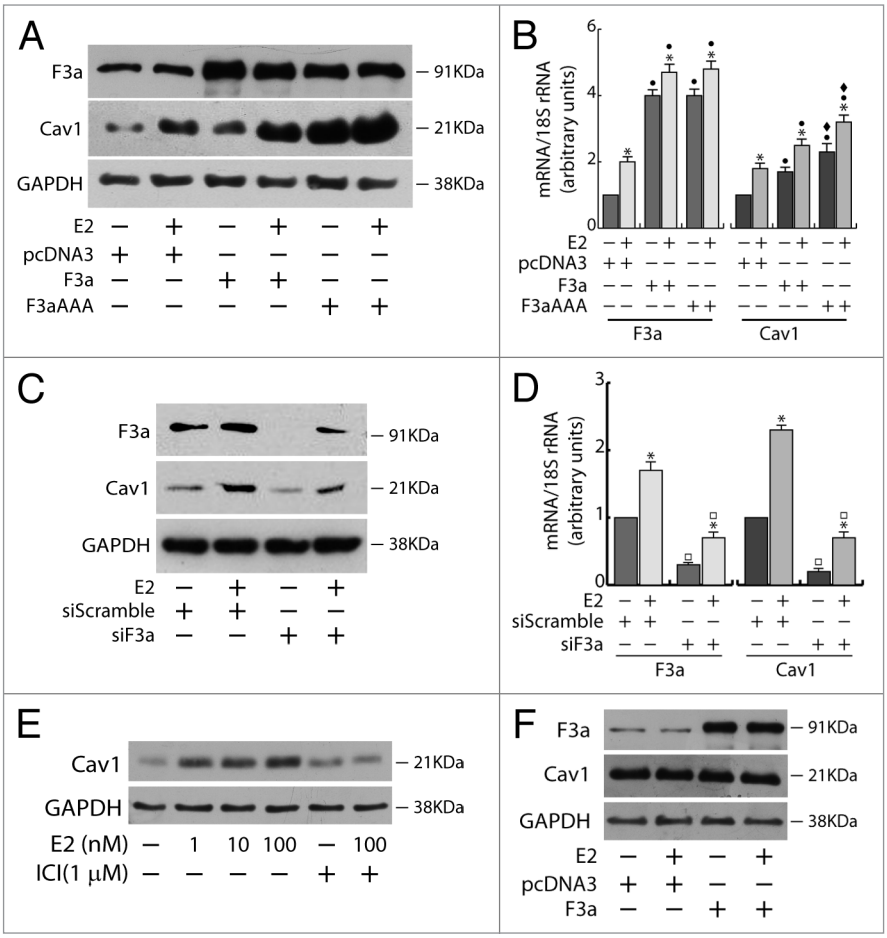


Figure 4. Cav1 expression depends on E2 and FoxO3a in ERα+ MCF-7 breast cancer cells. A double set of MCF-7 cells were either transiently transfected with F3a, F3aAAA, or pcDNA3 or silenced for FoxO3a, serum starved after 5 h and treated the next day with 100 nM E2 for 24 h. Cells were then harvested 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.

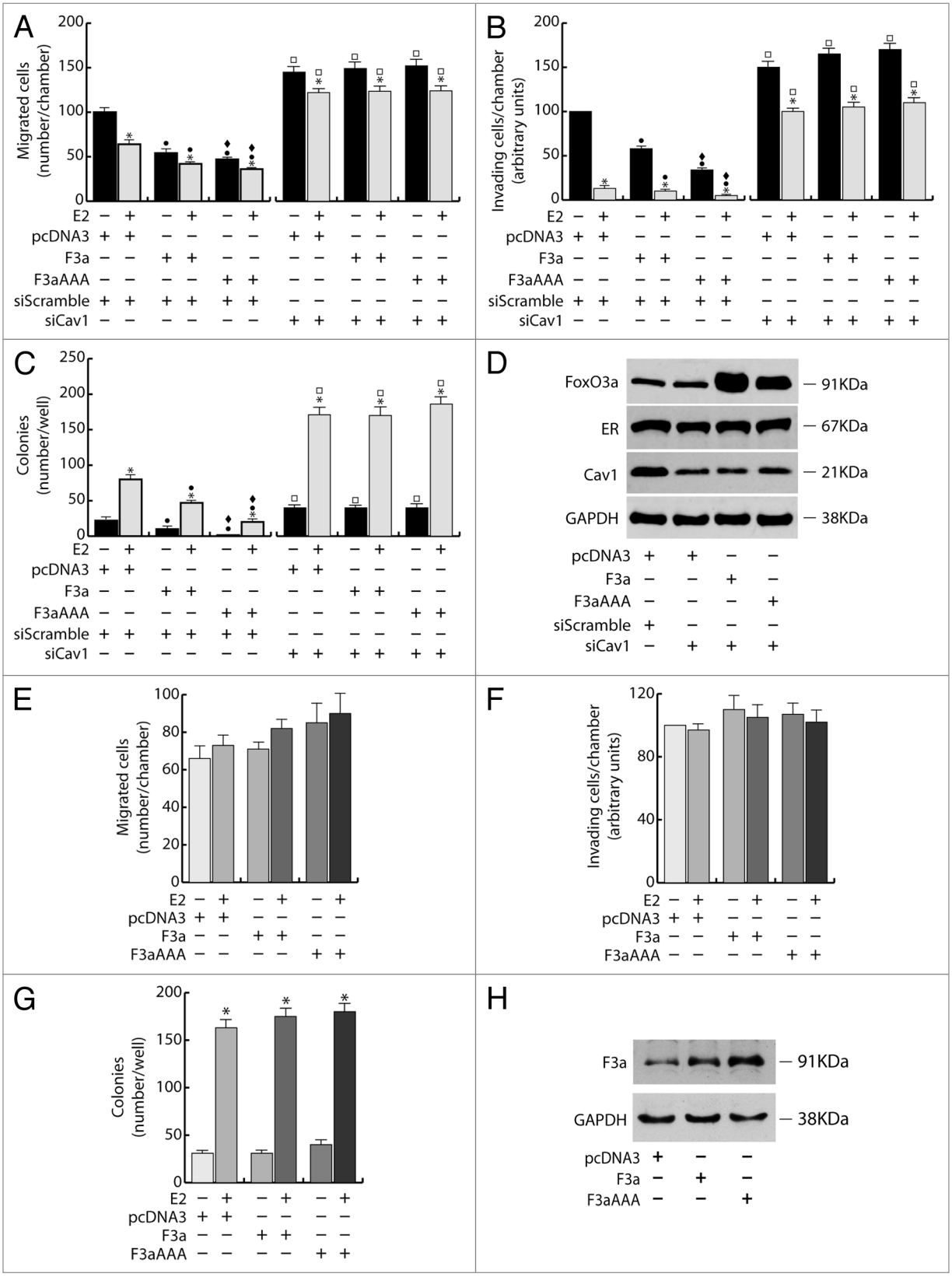


Figure 5. For figure legend, see page 3410.

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.

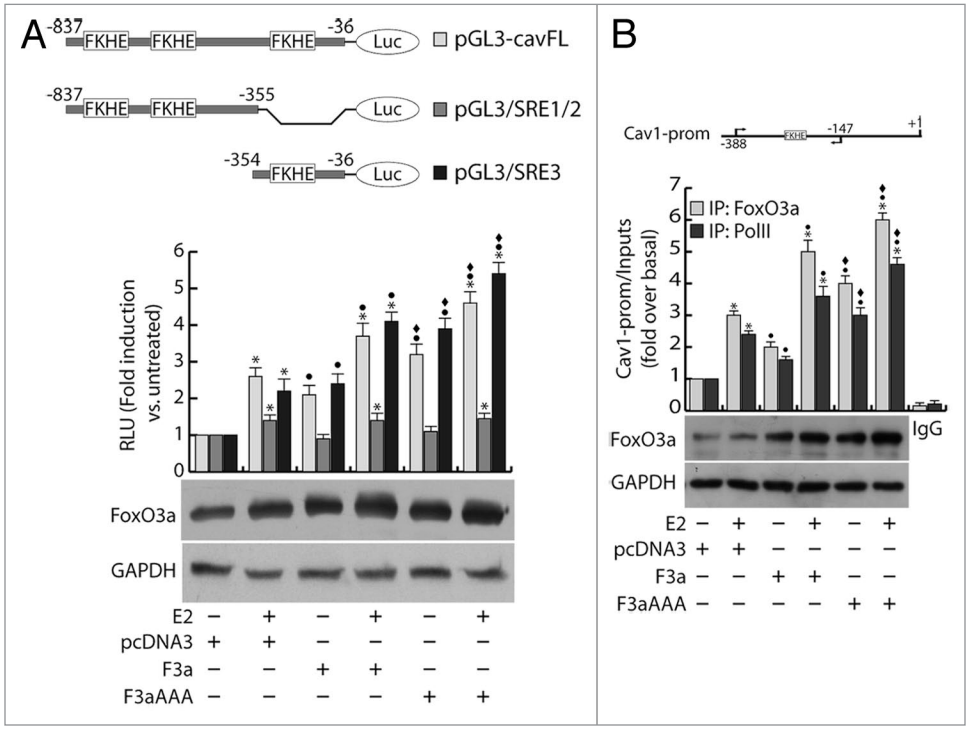


Figure 6. FoxO3a binds to and transactivates the Cav1 promoter. (**A**) MCF-7 were seeded in culture medium on 24-well plates, serum starved for 24 h, co-transfected in PRF-CT with pGL3-cavFL, or pGL3/SRE1/2, or pGL3/SRE3 and pRL-Tk, in presence of either pcDNA3 or F3a or F3aAAA vectors. After 6 h, E2 (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.

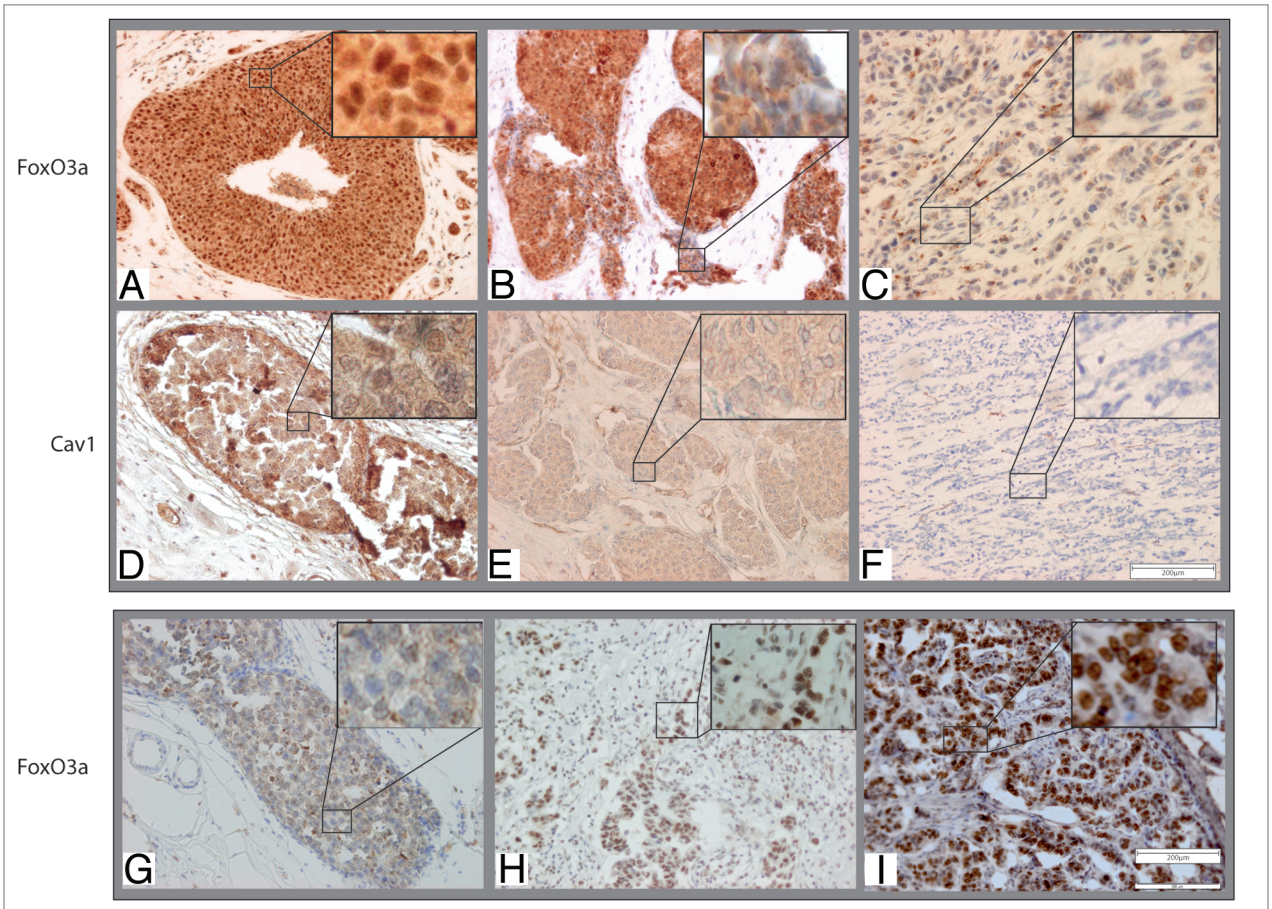
To prove our hypothesis, minimally motile and invasive ERα+ MCF-7 and ZR-75 breast cancer cell lines have been transfected with wild-type F3a and constitutively active F3aAAA mutant, and the effects on cell migration, invasion, and colony formation in soft agar were observed. The results presented here 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.

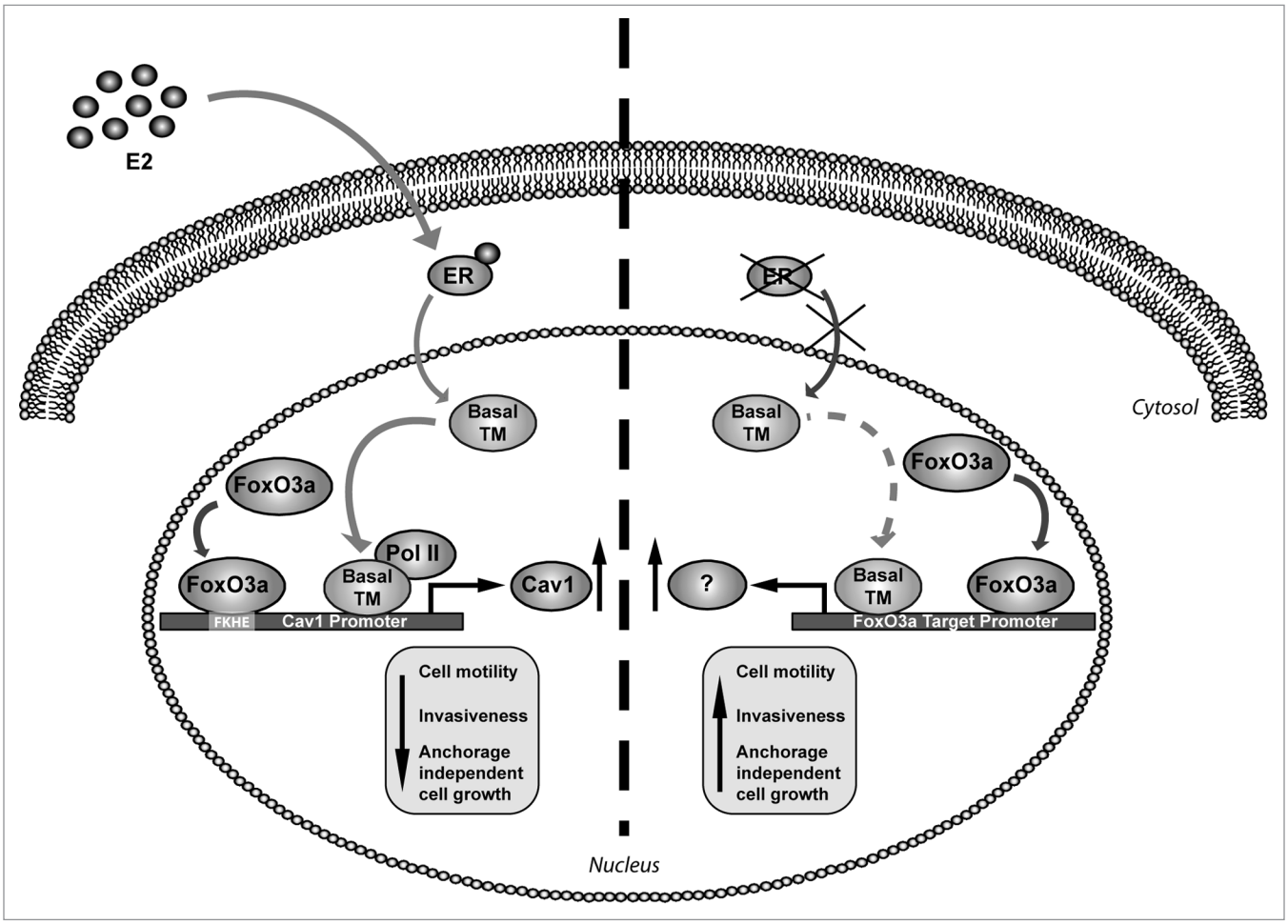


Figure 8. Proposed model for FoxO3a-mediated control of cell motility and invasiveness in presence or absence of ERα. F3a and ERα synergistically induce the expression of Cav1, which, in turn, reduces cell motility and invasiveness of ERα+ breast cancer cells. Transcriptionally active F3a binds to a 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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