UNIVERSITA' DELLA CALABRIA

Dipartimento di Farmacia e Scienze della Salute e della Nutrizione

Dottorato di Ricerca in

"Biochimica Cellulare ed Attività dei Farmaci in Oncologia"
(XXVII Ciclo)

Con il contributo della Commissione Europea, Fondo Sociale Europeo e della Regione Calabria

---

"Effects of adiponectin on the progression of breast cancer: role of Estrogen Receptor alpha"

MED 04/Patologia Generale

Coordinatore: Ch.mo Prof. Diego Sisci

Tutor: Prof. Loredana Mauro

Dottorando: Dott.ssa Emilia Ricchio

Anno Accademico 2013/2014

"La presente tesi è cofinanziata con il sostegno della Commissione Europea, Fondo Sociale Europeo e della Regione Calabria. L'autore è il solo responsabile di questa tesi e la Commissione Europea e la Regione Calabria declinano ogni responsabilità sull'uso che potrà essere fatto delle informazioni in essa contenute".

INDEX

❖ **Abstract** 1

❖ **Adipose tissue and adipokines** 3

- Adipose tissue 3
- Adipokines 6
- Obesity and breast cancer 8
- Adiponectin 15

❖ **Estrogens and their receptors** 19

- Estrogens 19
- Estrogen Receptors 21
- Estrogen and breast cancer 26

❖ **Materials and Methods** 31

❖ **Results** 39

❖ **Discussion** 58

❖ **References** 64

Abstract

Adipose tissue is no longer considered an inert tissue for storing energy but is now recognized as an active endocrine organ secreting adipokines, cytokines and a diverse range of inflammatory markers.

Adiponectin is one of the adipokines secreted by white adipose tissue and has been suggested to improve insulin sensitivity, regulate glucose and lipid metabolism and might play a role in the development of diabetes and atherosclerosis.

In addition, it appears to play an important role also in the development and progression of several obesity-related malignancies, including breast cancer.

In the present study, we demonstrated that adiponectin induces a dichotomic effect on breast cancer growth. Indeed, it stimulates growth in ERα-positive MCF-7 cells while it inhibits proliferation of ERα-negative MDA-MB-231 cells. Notably, only in MCF-7 cells adiponectin exposure exerts a rapid activation of MAPK phosphorylation, which is markedly reduced when knockdown of the ERα gene occurred. In addition, adiponectin induces rapid IGF-IR phosphorylation in MCF-7 cells, and the use of ERα siRNA prevents this effect. Moreover, MAPK activation induced by adiponectin was reversed by IGF-IR siRNA.

Coimmunoprecipitation studies show the existence of a multiprotein complex involving AdipoR1, APPL1, ERα, IGF-IR, and c-Src that is responsible for MAPK signaling activation in ERα-positive breast cancer cells. It is well known that in addition to the rapid effects through non-genomic mechanisms, ERα also mediates nuclear genomic actions. In this concern, we demonstrated that

adiponectin is able to transactivate ERα in MCF-7 cells. We showed the classical features of ERα transactivation: nuclear localization, downregulation of mRNA and protein levels, and upregulation of estrogen dependent genes.

Finally we demonstrate that *in vivo* adiponectin (1 and 5 μg/ml) induces a significant reduction (60 and 40%, respectively) in tumor volume in animals injected with human ERα-negative MDA-MB-231 cells, whereas an increased tumor growth (54 and 109%, respectively) is observed in the animals receiving human ERα-positive MCF-7 cells. Moreover, cyclin D1 (CD1) mRNA and protein levels are decreased in MDA-MB-231 cells, while they are upregulated in MCF-7 cells by adiponectin.

Collectively, this study clarifies the molecular mechanism through which adiponectin modulates breast cancer cell growth, providing evidences on the cell-type dependency of adiponectin action in relationship to ERα status.

CAPITOLO I

I.1 Adipose tissue

Adipose tissue is a loose connective tissue located either subcutaneously just under the skin, in neck regions, or at several intra-abdominal, or visceral, locations inside the thorax and abdomen in close proximity to major internal organs (Fantuzzi G. &. Mazzone T, 2007).

In general, adipose tissue can be divided into two major types: white adipose tissue (WAT) and brown adipose tissue (BAT), (Fig. I-1).

Brown adipocytes are only found in mammals and have a multilocular distribution of triglycerides droplets and a vast number of specialized mitochondria which contain the uniquely BAT-expressed protein, uncoupling protein-1 (UCP1), which generates heat at the expense of ATP (Rosen E.D. &. Spiegelman B.M. ,2006; Cannon B. & Nedergaard J., 2004). Until recently, the existence of BAT was thought to be restricted to small mammals and infants, but has now been shown to be present also in adult humans (Virtanen K.A. et al., 2009; van Marken Lichtenbelt, W.D. et al., 2009).

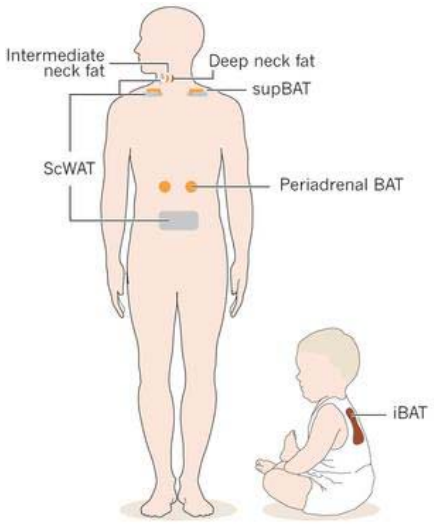

Fig I-1. Locations of the brown adipose tissue (BAT) and WAT depots in infants and adults. iBAT, interscapular BAT; supBAT, supraclavicular BAT; scWAT, subcutaneous WAT.

WAT is composed of mainly adipocytes and other distinct cell types including mature adipocytes, pre-adipocytes, fibroblasts and macrophages, all of which participate to a greater or lesser extent in WAT secretary functions. WAT is commonly called unilocular because most of the cytoplasm of adipocyte cells is occupied by a single large drop of fat, crushing the nucleus and mitochondria to one side of the cell (Hiragun A., 1985), (Fig.I-2).

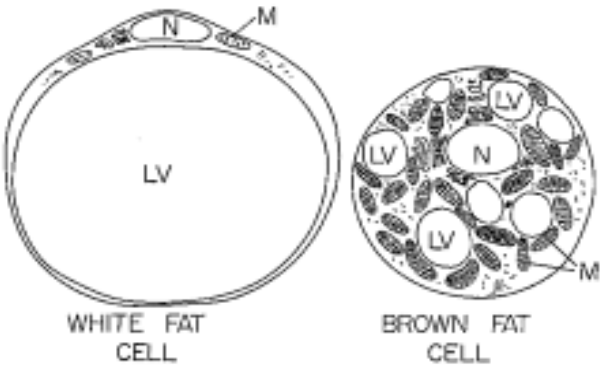

Fig I-2. White fat cell and brown fat cell: note the single large lipid vacuole in the white fat cell and the numerous smaller lipid vacuoles in the brown fat cell. LV: lipid vacuole; M: mitochondria; N: nucleus.

Adipose tissue is no longer considered an inert tissue mainly devoted to energy storage but is now recognized as an active endocrine organ secreting several hormones and a diverse range of other protein factors (Trayhurn P. & Wood I.S., 2004). The proteins secreted by adipocytes are called adipocytokines.

Leptin, adiponectin, vistafin, and resistin are a few of the well known adipocytokines. Macrophages in WAT also secrete a number of cytokines including interleukin-6 (IL-6), tumour necrosis factor alpha (TNFRα), interleukine-5 (IL-5), macrophage chemoattractant protein (MCP). All hormones and protein molecules secreted by adipose tissue are collectively called adipokines. The number and range of adiposity secretary proteins is continuing to

expand rapidly and approximately 50 different molecular entities have been identified so far (Trayhurn P. & Wood I.S., 2004).

Many of these proteins and factors act as endocrine hormones, for example leptin and IL-6, while others act locally, for example the TNF-α and growth factors. The proteins or endocrine factors are proposed as modulators of the function of different organs, such as the liver, skeletal muscle or brain (Fig.I-3).

Even paracrine/autocrine factors can affect insulin sensitivity, promoting or inhibiting the proliferation and/or cellular differentiation of adipocytes (Su A. I. et al., 2004).

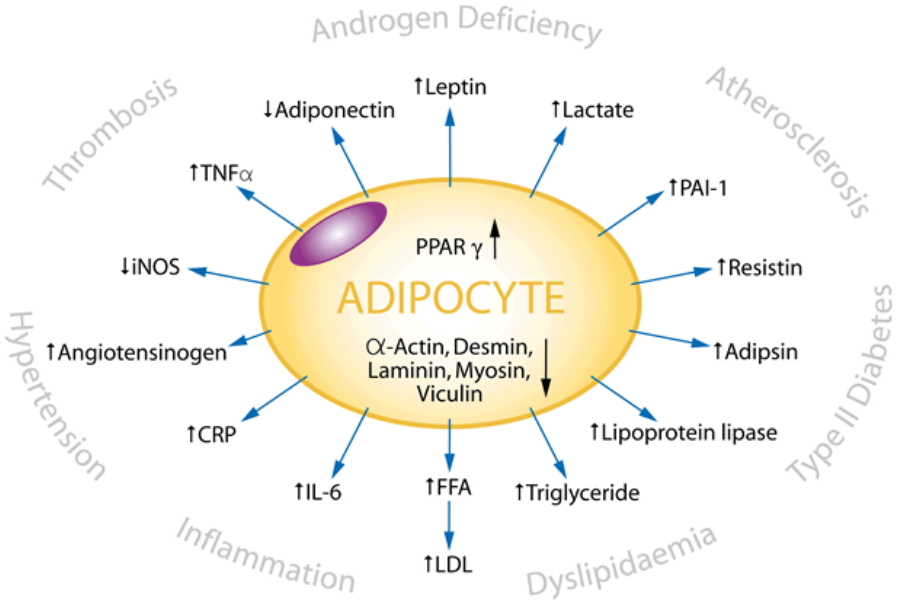

Fig I-3. Main factors secreted by adipocytes and their metabolic functions

I.2 Adipokines

Adipokines, proteins derived from white adipose tissue, play a very important role in the homeostasis of the organism, since they are capable of affect a wide variety of biological and physiological processes: food intake, the regulation of energy balance, the action of insulin, glucose and lipid metabolism, angiogenesis and vascular remodelling, regulation of the blood pressure and coagulation (Frühbeck G. et al., 2001).

Currently, more than 50 different adipocytokines have been identified, which mainly regulate energy metabolism, but also have several pathophysiological functions.

The adipokines can be classified into three groups:

1. Hormones produced primarily in other tissues or organs with simultaneous adipose tissue production (i.e. TNF α).

2. Hormones produced mainly in the white adipose tissue. Nevertheless, adipocytes are not the only source of production and other cells residing in fat, i.e. immunocompetent cells, may also participate (resistin).

3. Hormones produced predominantly or exclusively by adipocytes of white adipose tissue (leptin and adiponectin).

Another classification of adipocytokines reflects their putative physiological role. According to this classification, adipocytokines may be divided into two groups: "insulin resistance-inducing factors" such as resistin, TNF-α and interleukin 6, and "insulin-sensitizing factors" such as leptin, adiponectin and the recently described visfatin (Fukuhara A. et al., 2005), (Fig.I-4).

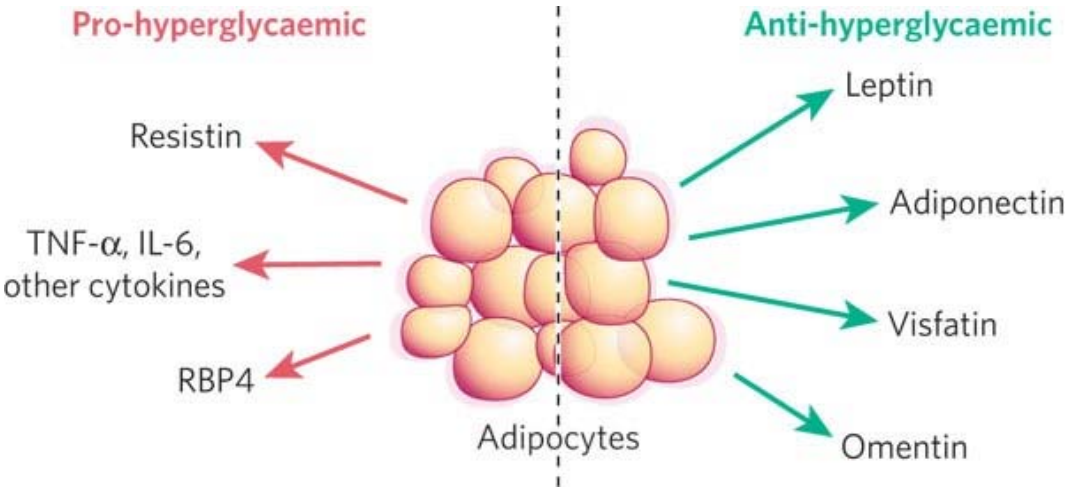

Fig I-4. Classification of adipokines

The well known adipokines (leptin, adiponectin, resistin) secreted by adipose tissue, are widely studied for their involvement in the mechanisms through which obesity and metabolic disorders affect the risk of carcinogenesis. It was therefore studied the direct and indirect effect of these protein factors in the biological and clinical development of breast cancer, emphasizing their different mode of action through endocrine, paracrine and autocrine system (Harvie M. et al., 2003; Rose D.P. et al., 2004). In addition to the relationship between adipocytokines and obesity or diabetes numerous other functions of these hormones in human body have been identified, including their potential role in the regulation of angiogenesis and tumor growth. Disturbances in the production of adipocyte-derived hormones thus may represent a new link explaining the well-known relationship between obesity and increased prevalence of malignancies.

I.3 Obesity and breast cancer

Breast cancer is the uncontrolled growth and spread of abnormal cells that initiates in mammary tissue. It often begins in the ducts, the tubes that drain milk from the breast, or in the lobules, the glands in the breast that produce milk (Argani P. & Cimino-Mathews A., 2012). There is no single known cause of breast cancer, and only 5 to 10% of these diagnoses are attributed to genetic mutations inherited from a parent.

Of these cases, BRCA1 and BRCA2 mutations are associated with 80% and 65% lifetime risk of breast tumour diagnosis, respectively.

Outside of genetics, several risk factors for the disease have been established: increased age, family history of breast cancer, early-age menarche, late-age menopause, late-age first live birth, extended use of hormone replacement therapy, alcohol consumption, and living a sedentary lifestyle (Hankinson S. et al., 2008).

Like all cancers, breast tumors are categorized with a high degree of diversity of clinical characteristics, disease pathologies and therapeutic responses.

"Carcinoma *in situ*" means that the cancer is still restricted to its tissue of origin.

There are two types of breast carcinoma *in situ*: lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS), (Fig.I-5). *In situ* breast cancers are treated with resection surgery and radiation therapy.

However, women with LCIS are at higher risk of having a future occurrence of invasive cancer in either breast, and untreated DCIS will likely grow into an invasive cancer (Argani P. & Cimino-Mathews A., 2012).

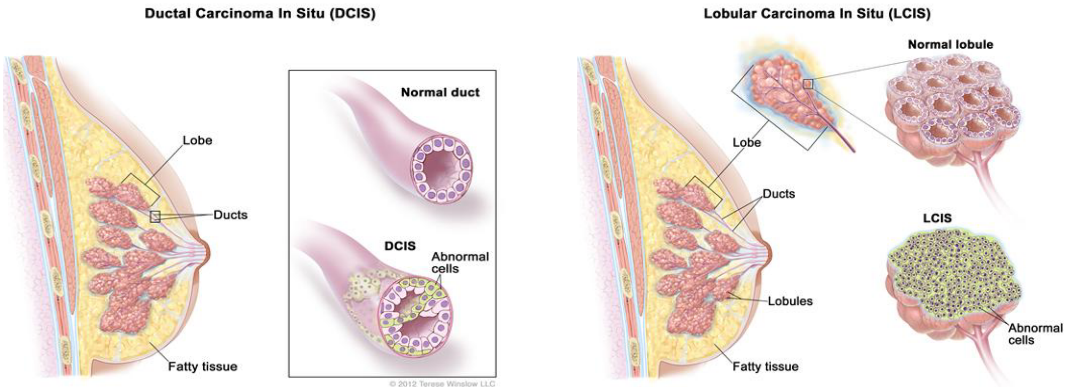

Fig I-5. Ductal carcinoma *in situ* (DCIS): abnormal cells are found in the lining of a breast duct. Lobular carcinoma *in situ* (LCIS): abnormal cells are found in the lobules of the breast.

Invasive cancers are those that have spread from the ducts and lobules and into other breast tissue, fatty tissue, or surrounding lymph nodes. Invasive ductal carcinomas (IDC) and invasive lobular carcinomas (ILCs) are the most frequent diagnoses of invasive breast cancer diagnosed with a 80% and 15% frequency, respectively (Fig.I-6). There are four subtypes of IDC: colloidal, medullary, metaplastic, and tubular carcinomas. Among these, both colloidal and tubular carcinomas have a better prognosis because of their lower probability of metastasis.

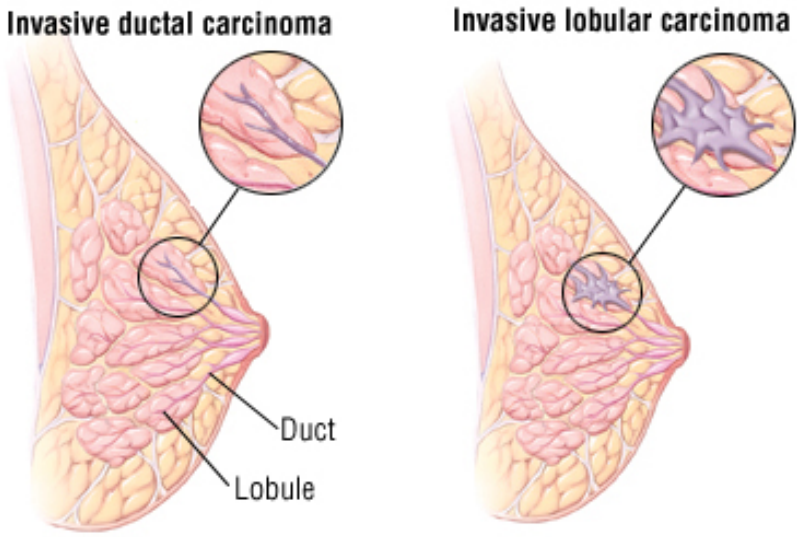

Fig I-6. Invasive ductal carinoma and invasive lobular carcinoma

A third, and extremely rare type of invasive breast cancer is inflammatory breast cancer (IBC) and occurs in 1 to 3% of breast cancer diagnoses (Argani P. & Cimino-Mathews A., 2012).

The pathogenesis of breast cancer is largely dependent on interactions between malignant cells and components of the breast microenvironment. The malignant cell phenotype is regulated not only by autonomous signals originating from cancer cells, but also by the effects of the surrounding stromal cells, which influence mammary epithelial cell growth and differentiation. The close association between mammary epithelial cells and adipocytes may promote a more direct action of adipokines on that tissue (Körner A., 2007).

Obesity is associated with an increase in white adipose tissue and greatly alters the local and systemic secretion of biologically active adipocytokines. This change in adipokine secretion represents a significant risk factor for the development of many types of cancers, such as breast cancer.

Due to the fact that pre- and postmenopausal breast cancers are essentially two different diseases, their connection with obesity is equally different. Several studies have identified either no or even an inverse relationship between obesity and premenopausal breast cancer (Stephenson G.D. & Rose, D.P., 2003). Reduced ovarian production of estrogen in premenopausal obese women has been suggested as a reason for this association (Abrahamson P.E. et al., 2006).

Obesity has however been suggested as a risk factor for premenopausal breast cancer because of the relationship between adiposity and ER-negative breast cancers (Rose D.P. & Vona-Davis L., 2009). Indeed Daling and colleagues (2001) found that, while premenopausal obese women do not have an elevated risk of developing cancer, obese premenopausal breast cancer patients had higher incidences of ER-negative, thus more malignant tumours, which resulted in longer treatments with less favourable outcomes. Hence obesity may not increase premenopausal breast cancer incidence rates, but may instead increase the severity of premenopausal breast cancer.

Obese postmenopausal women have an increased risk of developing breast cancer (Abrahamson P.E. et al., 2006; Lahmann P.H. et al., 2004). It has been estimated that women with a BMI over 40 kg/m^2 are more than twice as likely to develop breast cancer compared to women with normal BMI (Calle E.E. et al., 2003).

However, several molecular causes for the relationship between obesity and breast cancer have been suggested (Lorincz A.M & Sukumar S. 2006). First, obesity may increase the production of estrogen, which in turn can predispose to and promote progression of breast tumours (the estrogen connection). Second, obesity mediated hyperinsulinaemia may promote breast cancer, caused by the mitogenic

potential of insulin (the insulin connection). Third, obesity-related deregulation of adipokine secretion may promote breast cancer by adipocytes acting as mitogens or promoting an inflammatory state (the adipokine connection). It is unlikely that only one connection will be solely responsible for mediating the molecular connection. All three connections are probable contributors to the overall obesity-breast cancer relationship. Individually, one may outweigh the others, especially concerning the on-off contribution of the estrogen receptor. Lastly synergistic effects of these three connections may contribute to increase breast cancer risk (Fig.I-7).

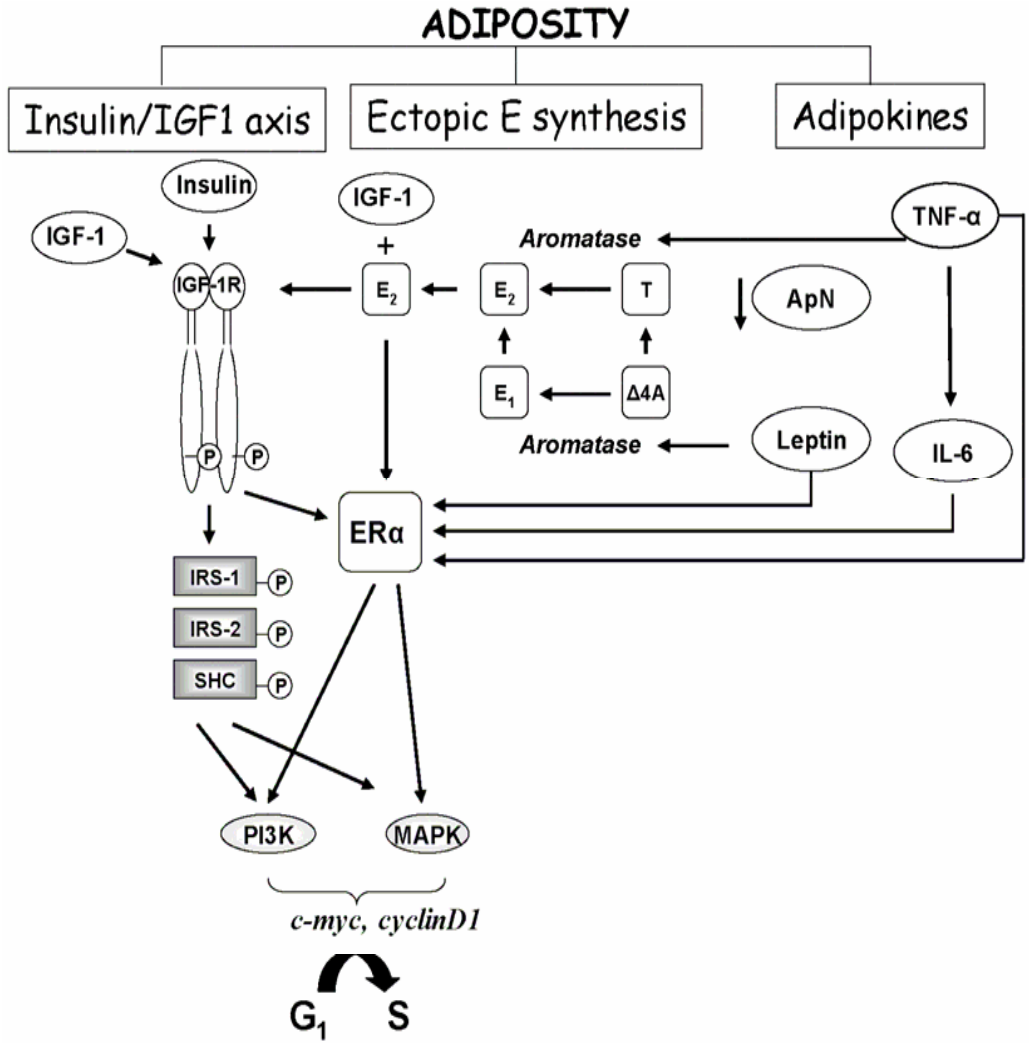

Fig I-7. Molecular mechanisms of correlation between obesity and breast cancer.

Adipokines (leptin, adiponectin, and hepatocyte growth factor (HGF)) secreted from adipose tissue have come to be recognized for their contribution to the mechanisms by which obesity and related metabolic disorders influence breast cancer risk.

Leptin is a peptide hormone of 16 kDa, synthesized and secreted by adipocytes of white adipose tissue. It is closely related to fat mass (Bjørbaek C. et al., 1997; Dieudonne M.N. et al., 2002). Leptin acts at the level of hypothalamic centers as a neuro-regulators of appetite, regulates thermogenesis and energy expenditure (Zhang Y., et al., 1994; Szanto I. et al., 1999).

It also reduces intracellular levels of lipids in skeletal muscle, liver and pancreatic beta cells, thus improving insulin sensitivity (Minokoshi Y. et al., 2002).

In addition to these functions, recent studies support a possible role of leptin in the development and/or progression of certain types of tumors. In particular, leptin has been proposed as a "link cytokine" between obesity and breast cancer (Garofalo C. & Surmacz E., 2006). Leptin is required for normal development of the mammary gland and lactation (Neville M.C. et al., 2002), but can also contribute to mammary tumorigenesis. In fact, many authors have shown the expression of the leptin gene both at the level of the normal and tumoral breast tissue.

Leptin stimulates growth, migration, and cell invasion of tumor cells *in vitro* and also enhances angiogenesis, manifesting its ability to promote the biological behaviour of tumor aggression. The role of leptin in mammary carcinogenesis is further supported by recent studies that have demonstrated that leptin directly transactivates estrogen receptor (ER)α (Catalano S. et al., 2004) and promotes *in situ*

estrogen production by increasing aromatase expression and activity in breast cancer cells (Catalano S. et al., 2003).

Another important adipokine that appears to be involved in the development of breast cancer is adiponectin.

In the mammary gland, epithelial cells are exposed to both circulating and locally produced adiponectin from adjacent adipocytes (Andò S. & Catalano S., 2012).

In contrast to leptin, epidemiological studies have demonstrated an inverse association between the serum adiponectin levels and breast cancer risk.

Particularly, breast tumors arising in patients with hypoadiponectinemia may have a more aggressive phenotype (large tumor size, high histological grade, estrogen receptor negativity, and increased metastasis), (Miyoshi Y. et al., 2003; Mantzoros C. et al., 2004). Recently it has been reported that low circulating adiponectin levels are associated with a higher risk of breast cancer development, and that this association is independent of age, BMI, and hormonal and reproductive. Other studies described a significant negative correlation between BMI and plasma adiponectin levels in both men and women and that adiponectin levels are negatively correlated with percent body fat, waist-to-hip ratio and intra-abdominal fat (Weyer C. et. al., 2001; Andò S. & Catalano S., 2012).

The secretion of adiponectin into the bloodstream is not regulated by subcutaneous but rather by visceral adipose tissue (Körner A. et al., 2007).

I.4 Adiponectin

Adiponectin is a protein of molecular weight 30 kDa produced exclusively in white adipocytes. The molecule of human adiponectin consists of 244 amino acid residues; at the N-terminus there is an 18 amino acid long signal peptide followed by a short hypervariable region without homology to any known sequences and a collagen domain with 22 repeated motifs. C-terminal contains globular domain homologous to C1q molecule of complement cascade. C-terminal globular domain also shows homology with TNF-α trimeric cytokines family.

Adiponectin molecules are secreted from adipocytes as trimers (~90 kDa; the basic unit), low molecular weight hexamers (~180 kDa) and high molecular weight isoforms (12-18-mers; >400 kDa) (Fruebis J. et al., 2001).

In serum, adiponectin exists in its full-length form (fAdiponectin) that requires post-translational modifications for activity (e.g. hydroxylation and glycosylation), (Fig. I-8).

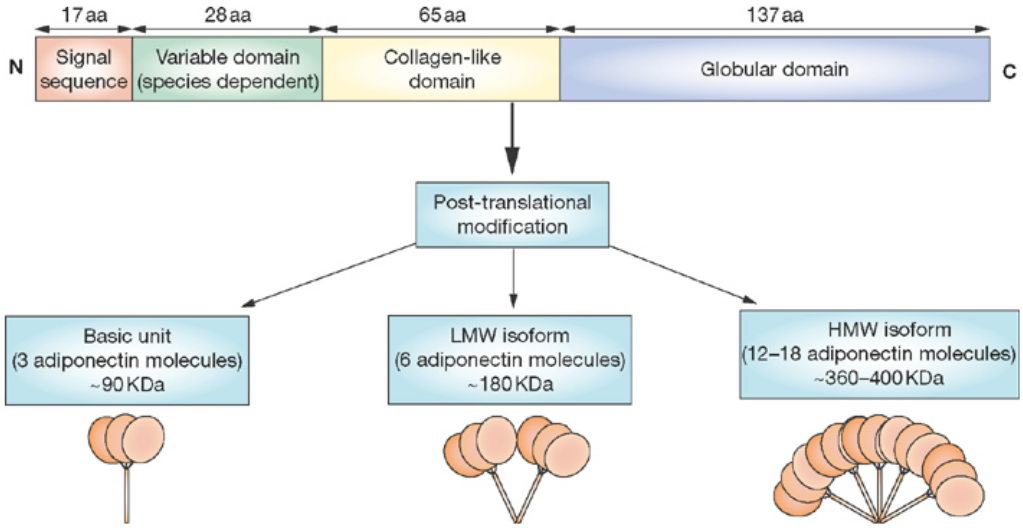

Fig I-8. Structure of adiponectin

The cellular functions of adiponectin are mediated through the adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) and T-cadherin (Brochu-Gaudreau K. et al., 2010). AdipoR1 and AdipoR2 are integral membrane proteins containing 7 transmembrane domains but are structurally and functionally distinct from G protein-coupled receptors (Yamauchi T. et al., 2003). AdipoR1 presents high affinity for gAdiponectin and low affinity for fAdiponectin, and it is expressed ubiquitously but abundantly in skeletal muscle and endothelial cells. AdipoR2 has intermediate affinity for both forms of adiponectin and is predominantly expressed in the liver (Kadowaki T. & Yamauchi T., 2005). It has been demonstrated that the globular form of adiponectin binds to AdipoR1 (Yamauchi T. et al., 2003), which, in turn, through the adaptor protein APPL1 interacting with the intracellular N terminus of AdipoR1, induces activation of MAPK through Src pathway (Mao X. et al., 2006; Lee M-H. et al., 2008). APPL1, which contains a pleckstrin homology domain, a phosphotyrosine- binding domain, and a leucin zipper motif, has emerged as an important element in AdipoR1/R2 signaling (Mao X. et al., 2006), (Fig.I-9).

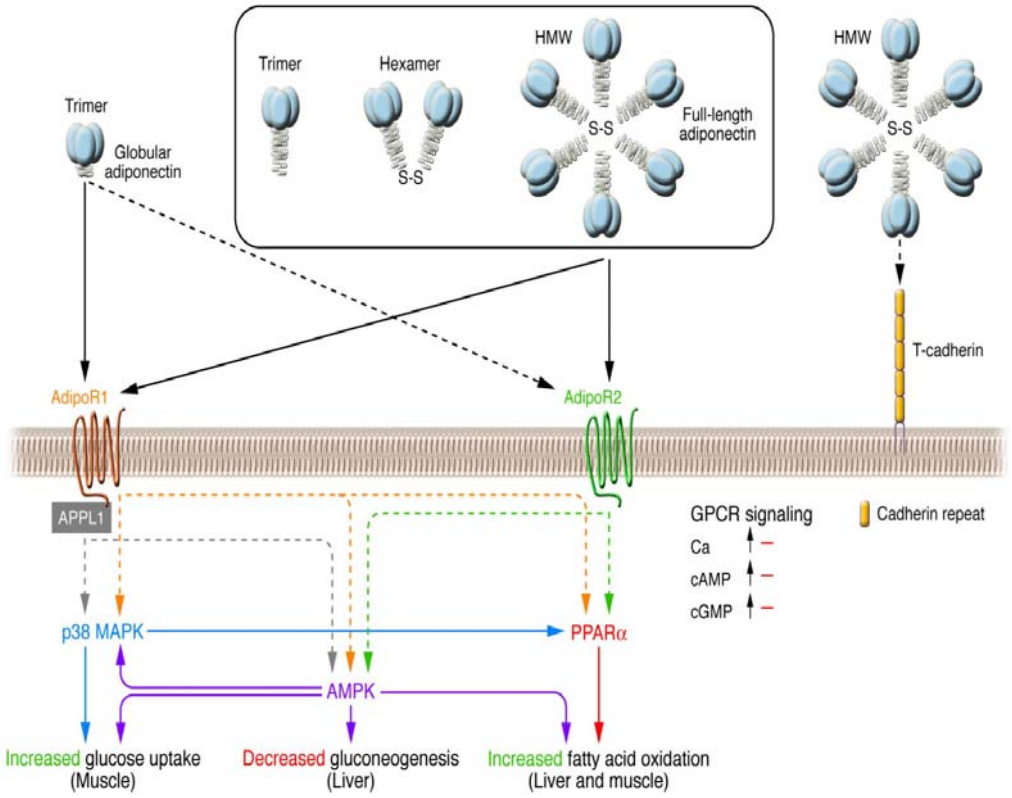

Fig I-9. Signal transduction by adiponectin receptors

Adiponectin has a protective role against obesity-related disorders, including metabolic syndrome, type-2 diabetes, and cardiovascular disease (Brochu-Gaudreau K. et al., 2010). Unlike most of the other adipokines, serum adiponectin is inversely correlated with body mass index (BMI) (Matsubara M. et al., 2002; Cnop M. et al., 2003). Circulating adiponectin levels are reduced in obesity and type 2 diabetes (Weyer C. et al., 2001) and mice lacking adiponectin develop insulin resistance, glucose intolerance, hyperglycemia, and hypertension, all characteristics of metabolic syndrome (Kubota N. et al., 2003; Maeda N. et al., 2002).

A speculative explanation of the reduced adiponectin levels in obesity may be sustained by the enhanced production of cytokines that occurred in such

condition, and may contribute to the downregulatory effect on adiponectin secretion by adipose tissue (Tilg H. et al., 2006).

Another potential mechanism indicates a negative feedback of adiponectin on its own production and probably on the expression of its receptors during the development of obesity (Dalamaga M. et al., 2012). Recent experimental and clinical investigations suggested that low levels of plasma adiponectin are associated with an increased risk for obesity-related cancers, such as colon, prostate, endometrial, and breast cancer (Dalamaga M. et al., 2012; Kelesidis I. et al., 2006; Miyoshi Y., 2003).

CAPITOLO II

II.1 Estrogens

Estrogens are the primary female sex hormones and play important roles in both reproductive and non-reproductive systems. The major sources of estrogens are the ovaries and the placenta (the temporary organ that serves to nourish the fetus and remove its wastes); additional small amounts are secreted by the adrenal glands and by the male testes.

Cholesterol is the parent molecule from which all ovarian steroid hormones are formed. Cholesterol is converted to pregnenolone, and pregnenolone is converted to progesterone. The steps in the conversion of progesterone to the main estrogens (estradiol and estrone) include the intermediate formation of several androgens (male sex hormones): dehydroepiandrosterone, androstenedione, and testosterone. Androgens are precursors of estrogens; they are converted to estrogens through the action of an enzyme known as aromatase. The ovaries are the richest source of aromatase, although some aromatase is present in adipose tissue, which is also an important source of estrogen in postmenopausal women. Estradiol, the most potent estrogen, is synthesized from testosterone. Estrone can be formed from estradiol, but its major precursor is androstenedione. Estriol, the weakest of the estrogens, is formed from both estrone and estradiol (Fig.II-1).

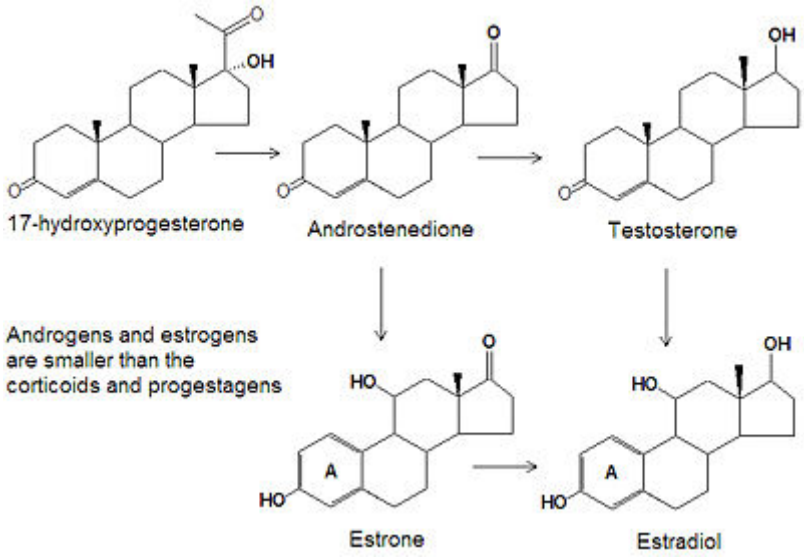

Fig.II-1 Estrogen synthesis

Estrogens are essential for the correct functioning of the reproductive system female and are essential for the proliferation and differentiation of normal breast. In fact, the reduction of estrogen levels, found in women after menopause, is associated with an increased risk of developing malignant breast. The estrogenic compounds also have beneficial effects on the heart and bone structure of the human organism, maintaining bone density and reducing the risk of fractures (Watanabe T. et al., 1997). Several studies showed that these hormones affect the centers of brain responsible for the maintenance and regulation of body temperature; they also play an important role in the central nervous, skeletal and cardiovascular systems.

Moreover, the estrogens are mainly responsible for the development of female secondary sexual characteristics, regulate the menstrual cycle and the pregnancy.

II.2 Estrogen Receptors

Natural human estrogens exert their biological effects via interaction with the two different isoforms of estrogen receptors, ERα, and ERβ. Both receptors belong to the superfamily of nuclear receptors, but are synthesized by different genes and possess peculiar structure and functions. The two isoforms are constituted by six domains (Edwards D.P., 2005), (Fig.II-2) and show high degree of sequence homology (96%) in region that binds DNA, (DBD), but differ considerably in the N-terminal domain and in the AF-1 (activation function) and to a lesser extent in the LBD (53%).

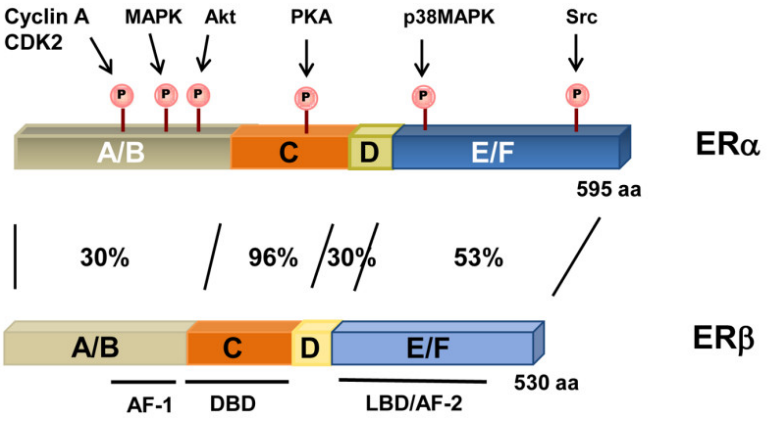

Fig II-2. Estrogen receptors structure

ER contains two "activation functions" (AF) that interact with coactivators. AF-1 ligand-independent is within the N-terminal domain, whereas for AF-2 that is in the ligand binding domain (LBD), its activity is dependent on ligand-induced conformational changes. The relative contribution of each AF is cell and promoter dependent.

The DNA-binding domain (domain C) is highly conserved between ERα and ERβ and contains the protein domains responsible for the specific binding to DNA at sites characterized by the sequence AGGTCA. In the estrogen receptor, this domain consists of a sequence of about 60 amino acids.

The next domain, D (hinge region), is a less conserved and particularly flexible, containing sequences for the nuclear translocation.

The domain larger, moderately conserved, is the domain E or LBD (Ligand-Binding-Domain), responsible for the interaction with the ligand.

The mechanism of activation of ER starts with the interaction of the receptor with its ligand. This binding induces a conformational change in the structure of the receptor with the consequent detachment of protein stabilizers that are associated with receptor (Fig. II-3).

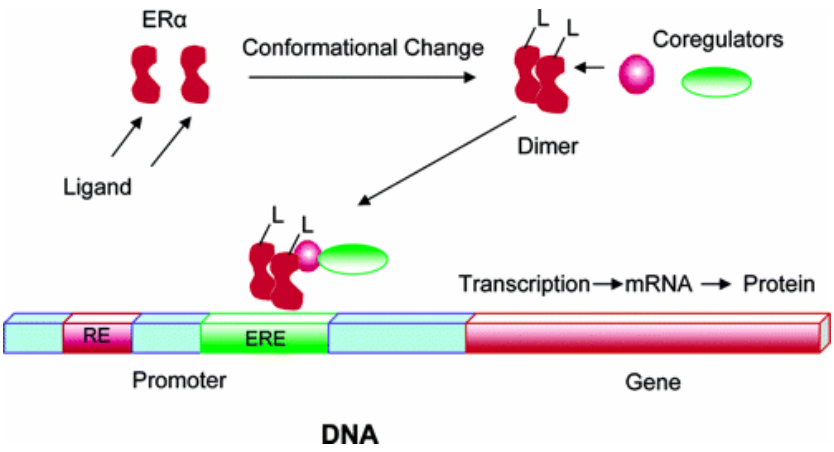

Fig II-3. Ligand-receptor interaction and activation of transcription

The hormone-receptor complex translocates to the nucleus and interacts with the sequences "estrogen-responsive" (ERE, Estrogen Responsive Elements). These regions are palindromic DNA sequences of 15 nucleotides consisting of a repetition, around a center of symmetry, of a same sequence reversed and

inverted. The sequence is recognized AGGTCAnnnTGACCT, in which the center of symmetry is composed of three nucleotides (nnn), (Clementi F. & Fumagalli G., 1999).

ERE sequences act as "enhancers" and amplify the transcription of genes downstream of palindromes, which code for proteins involved in physiological proliferative events (DNA replication, mitosis) or pathological (cancer development), (Lottering M.L. et al., 1992).

In addition to classic ERE-mediated activation mechanism, described above, are known other mechanisms that mediate the action of estrogen. One of these involves the activation of the estrogen receptor in a ligand-independent manner. In fact, growth factors such as IGF-I and EGF, are able to activate a pathway of intracellular kinases (MAPK) that induce the phosphorylation and the activation of ER, independently from the interaction with the ligand. The target domain of these phosphorylation events is the NH2-terminal in which the serine 118 (Ser 118) is often phosphorylated.

In some cell lines was identified a membrane estrogen receptor α whose activation occurs through a mechanism not genomic. In this case, estradiol binds to its membrane receptor determining the activation of complex transduction pathways and an immediate response in target tissues (Fig.II-4).

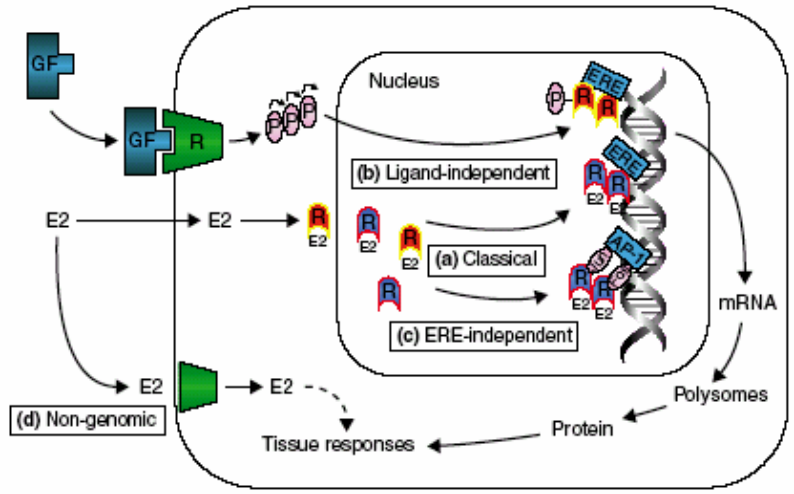

Fig. II-4. Mechanisms of activation of the estrogen receptor

ER ligands interact with ER subtypes in various parts of the human body (Fig.II-5). The abundance and distribution of the receptors will, in part, determine whether a ligand will have a particular effect. ERα and ERβ are known to be localized in the breast, brain, cardiovascular system, urogenital tract and bone (Pearce S.T. &. Craig Jordan V., 2004). ERα is the main ER subtype in the liver, whereas ERβ is the main ER in the colon. ERα and ERβ may also localize to distinct cellular subtypes within each tissue. For example, within the ovary, ERα is largely present in the thecal and interstitial cells, whereas ERβ is predominantly in the granulosa cells (Hiroi H. et al., 1999; Sar M. & Welsch F., 1999). In the prostate, ERβ localizes to the epithelium, whereas ERα localizes to the stroma (Weihua Z. et al., 2002).

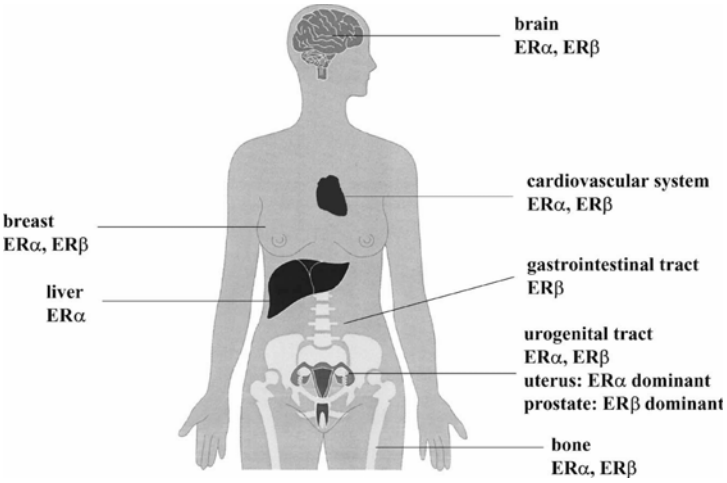

Fig. II-5. Distribution of ERα and ERβ in the human body

Estrogen Receptor alpha

ERα was the first estrogen receptor cloned and it was isolated from MCF-7 human breast cancer cells in the late 1980s (Walter P. et al., 1985; Greene G.L. et al.,1986; Green S. et al., 1986). In accordance with its role as a transcription factor, this 66 kDa ERα, encoded by ESR1 gene on chromosome 6q25.1, localizes primarily to the nucleus. A 46 kDa isoform (hERα46) that lacks the first 173 amino acids of the 66 kDa form of ERα has also been preliminarily characterized (Flouriot G. et al., 2000). In addition, several ERα splicing variants have been described (Murphy L.C. et al.,1997; Poola I. et al., 2000), but whether they are expressed as proteins that have a biological function remains unknown. Another source of variability in receptor function, and perhaps also dysfunction, is ERα gene polymorphisms. ERα polymorphisms have been linked to increased litter size in pigs (Short T.H. et al., 1997), breast cancer susceptibility (Andersen T.I. et al., 1994), bone mineral density and osteoporosis

(Mizunuma H. et al., 1997), hypertension (Lehrer S. et al., 1993), spontaneous abortion (Schachter B.S. & Lehrer C., 1994), and body height (Lehrer S. et al., 1994).

II.3 Estrogens and breast cancer

Epidemiological and experimental evidences implicate estrogens in the aetiology of breast cancer. Most established risk factors for breast cancer in humans are thought to influence risk through hormone-related pathways (Pike M.C. et al., 1993), increased concentrations of endogenous estrogens are strongly associated with increased risk for breast cancer in postmenopausal women (Cuzick J. et al., 2003), and trials have shown that the anti-estrogens tamoxifen and raloxifene reduce the incidence of breast cancer (Dao T.L., 1981). Furthermore, experimental studies in animals have shown that estrogens can promote mammary tumours, and a decrease in exposure to estrogens, by performing an oophorectomy or giving an anti-estrogenic drug, has the reverse effect (Henderson B.E. et al., 2000). However, the effects of estrogen alone do not fully account for the relationships observed between breast cancer and hormone-related risk factors. In many cases, this type of pathology, as reported , is hormone-dependent.

Estrogens are essential compounds for the growth of mammary cell that has undergone neoplastic transformation. The development and growth of the tumor are stimulated by estrogen, which exert a mitogen action on cells of the mammary gland (Dickson R.B. & Lippman M.E., 1988): transformed cells send wrong signals to the surrounding cells and estrogen excess products are able to induce a mitogenic effect on the surrounding tissue.

Therefore, through the interaction with its receptors, these steroid hormones are able to stimulate the proliferation of normal and transformed cells through the induction of proteins involved in nucleic acid synthesis and the activation of genes that regulate cellular division.

The rise of cell proliferation may increase the possibility of errors in DNA repair with the result of an accumulation of mutations. All of this can contribute to the modification of a normal cell into a cell hyperplastic up to the neoplastic transformation.

In addition to estrogens, other hormones may contribute to breast cancer, for example, progesterone, androgens, glucocorticoids, prolactin, thyroid hormones insulin, IGF-1, IGF-2, fibroblast growth factor (FGF) and epidermal growth factor (EGF), (Osborne C.K. & Arteaga C.L., 1990; Evans R.M., 1988). In normal conditions, the interaction of growth factors, cytokines and hormones with specific membrane receptors activates a cascade of intracellular signals, rising the activation and/or inactivation of numerous and specific genes (Wahli W. & Martinez E., 1991; Eerola H. et al., 2002).

Before menopause most circulating estrogen is periodically produced by ovaries, according to menopausal status. Estrogen concentrations increase in response to luteinising hormone and follicle stimulating hormone secreted by the pituitary gland and decrease in response to progesterone secreted by the corpus luteum (Nussey S.S. & Whitehead S.A., 2001). It stimulates differentiation of the endometrium and sexual drive, preparing the female body for the reception of a fertilised oocyte. Menopause is characterised by cessation of the menstrual cycle, due to

termination of the delicate interplay of oscillating reproductive hormones, including estrogen.

In postmenopausal obese women adipose tissue is the main source of estrogen, in form of estrone, and serum estrogen concentrations are directly related to BMI (Grodin J.M. et al., 1973). This increase in estrogen concentrations with increased BMI is caused by an overexpression of the aromatase enzyme in stromal adipose tissue cells (Cleland W.H. et al., 1985).

Breast cancer risk is increased with increased exposure to estrogen. Estrogen exposure is measured by age at menarche and menopause, hormone replacement therapy and the use of estrogen containing contraceptive (Feigelson H.S. & Henderson B.E., 1996). Cohort studies have positively linked estrogen concentrations with breast cancer risk (Berrino F. et al., 1996; Toniolo P.G. et al., 1995). Exposure of healthy breast epithelial cells to estrogen increased their potential to become malignant (Dickson R.B. & Lippman M.E. 1986). Furthermore breast cancers of obese postmenopausal women are more often ER-positive (Rose D.P. et al., 2004).

The exact actions by which estrogen predisposes to breast cancer development and contributes to breast cancer progression are not entirely known. Estrogen increases cell proliferation in healthy epithelial cells and ER-positive breast cancer cells (Dickson R.B. & Stancel G.M., 2000). Thus anti-estrogens and ER-inhibitors decrease growth of ER-positive breast tumours and are used in treatment of these tumours (Pearson O.H. et al., 1982). Since breast tissue is composed of up to 90% of adipose tissue, a direct cross-talk between adipose cells and breast cancer cells has been suggested (Bulun S.E. et al., 1994). Furthermore cancer cells

may cross-talk to the surrounding adipose tissue to produce more estrogen or even produce it themselves (Brodie A. et al., 1997; Chen S. et al., 2002).

Although risk for breast cancer increases with age, there is a marked decline in the rate of increase in risk with age following the loss of ovarian function, either as a result of a bilateral oophorectomy or due to the menopause (Hunter D.J. et al., 1997; Helmrich G. et al. 1983), showing that hormone production by the ovaries is a crucial risk factor for breast cancer in humans.

Epidemiological studies have also firmly established associations between risk for breast cancer and other reproductive factors, including nulliparity (having no children) or low parity, late age at first birth, and breast feeding.

A mechanism involving estrogens, and probably other hormones, has been proposed to explain both the transient increase in risk and the reduced risk in the long term associated with pregnancy. The very high serum levels of estrogens and progesterone during pregnancy stimulate growth of the mammary epithelium and also promote the differentiation of epithelial tissue, reducing the number of epithelial structures most vulnerable to malignant transformation. Thus, the short-term effect of pregnancy may be to promote the growth of cancer if a malignant transformation is present in the breast, but in the longer term the risk for breast cancer is reduced. In contrast, malignant transformations are more likely to have accumulated in the breast tissue of older women, and there might therefore be a higher risk of cancer developing in these women when breast cells are stimulated to divide during pregnancy. The effect of age at first birth highlights the importance of timing of exposure as a critical determinant of the effects of steroid hormones such as estrogen. Breast-feeding is associated with a modest decrease in

risk for breast cancer, above and beyond that associated with multiple pregnancies (4% for every 12 months of breast feeding). This effect might be due to the suppression of ovulation, reducing exposure to ovarian hormones (Ewertz M. et al., 1990).

MATERIALS AND METHODS

III.1 Cell culture

Cells utilized in the studies were obtained from American Type Culture Collection, where they were authenticated, stored according to supplier's instructions, and used within 4 months after frozen aliquots resuscitations.

MCF-7, T47D, and HeLa cells were cultured as described (De Amicis F. et al., 2013). MDA-MB-231 were maintained in DMEM/F-12 containing 5% fetal bovine serum (Sigma). SKBR-3 breast cancer cells were cultured in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS). Before each experiment, cells were grown in phenol red-free media, for at least 24 h and then treated with 1 and 5 μg/ml recombinant human gAdiponectin/ gAcrp30 (R&D Systems) as described.

III.2 Cell proliferation assays

MTT assays: after 3 days of treatment, cell proliferation was assessed by MTT assay as reported (Rovito D. et al., 2013) and expressed as fold change relative to vehicle-treated cells. Soft agar growth assays and anchorage-independent growth assays were conducted as previously described (Panno M.L. et al., 2012). Data represent 3 independent experiments performed in triplicate.

Three-dimensional spheroid culture and cell growth assays: for three-dimensional cultures, MCF-7 cells plated on 2% agar- coated plates were untreated or treated with adiponectin 1 and 5 μg/ml. After 48 h, three-dimensional cultures were photographed using a phase-contrast microscope (Olympus), and the cell numbers were evaluated as previously reported (Mauro L. et al., 2007).

III.3 RNA silencing

MCF-7 cells were transfected with RNA duplex of stealth siRNA targeted for the human ERα (SI02781401), IGF-IR (SI01074017), or APPL1 (GS26060) mRNA sequence or with a control siRNA (Qiagen, Milan, Italy) that does not match with any human mRNA, used as a control for non-sequence-specific effects. Cells were transfected using RNAiFect Transfection Reagent (Qiagen) as recommended by the manufacturer with minor modifications (Guido C., et al., 2012). After 5 h the transfection medium was changed with serum-free medium, and then the cells were exposed to treatments.

III.4 Western blotting and immunoprecipitation

Cells were lysed in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (aprotinin, PMSF, and sodium orthovanadate). The protein content was determined using Bradford dye reagent (Bio-Rad). Equal amounts of total protein were resolved on SDS-polyacrilamide gels and transferred onto a nitrocellulose membrane as described (Mauro L. et al., 2009). Blots were incubated overnight at 4°C and probed with the specific primary antibodies. Immunoblotting was performed as reported (Guido C., et al., 2012). For immunoprecipitation, 500 μg of total protein lysates were precleared for 1 h with protein A/G-agarose (Santa Cruz Biotechnology), incubated with primary Abs at 4°C for 18 h in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 0.1 mM Na3VO4), and then the antigen-Ab complexes were precipitated with protein A/G agarose for 2 h in HNTG buffer.

The immunoprecipitated proteins were washed 3 times with HNTG buffer, separated on SDS-PAGE, and processed by western blotting. The following antibodies were used: AdipoR1, ERα, c-Src, APPL1, EGFR, Cyclin D1 and GAPDH from Santa Cruz Biotechnology; pERα Ser118, pERαSer167, total MAPK and phosphorylated p42/44 MAPK (Thr202/Tyr204), IGF-IR, and pIGF-IR Tyr1131 from Cell Signaling Technology.

III.5 Kinase activity of c-Src

To assay for c-Src kinase activity MCF-7 cells were grown in PRF-SFM for 24 h and stimulated with 1 or 5 μg/ml adiponectin for 15 min. Cells were then lysed with RIPA lysis buffer (500 mM TRIS-HCl, 150 mM NaCl, 1% Triton X-100) containing 10 mM PMSF, 1.5 mg/ml aprotinin, and 2 mg/ml leupeptin and immunoprecipitated. A Protein G-agarose and an anti-APPL1 antibody complex were prepared to immunopurify the lysates. A measure of 1 μg of rabbit polyclonal anti-APPL1 antibody and 30 μl of protein G-agarose (Santa Cruz, Biotechnology) were incubated at 4°C for 1 h in 500 μl of PBS with a tube rotator. The complexes were microfuged and washed with 1 ml of lysis buffer for 3 times. At the end, 1000 μg of each cell lysates were added to the Protein G-agarose/anti-APPL1 antibodies and incubated at 4°C for 2 h rotating. The proteins/complexes were centrifuged and washed 3 times with the kinase buffer (200 mM PIPES, 100 mM MnCl2). c-Src kinase activity was assayed by a standard *in vitro* kinase assay using acidified enolase as substrate. The incubation was performed in a total volume of 50 μl composed of the immunoprecipitates and the kinase buffer containing 5 mM ATP, 1 μC of [γ32P]ATP, and 2.5 μg of acid denatured rabbit

muscle enolase (Sigma) as exogenous substrate. Samples were incubated at 30°C for 10 min then reduced with an equal volume of 2× SDS Laemmli sample buffer (Sigma) and aliquots of them (40 µl) were submitted to SDS-PAGE (acrylamide 11%). The dried gel was exposed to X-omat film (Kodak) for 12 h. The gels were stained with Coomassie blue to ensure that an equal amount of enolase was present in all samples.

III.6 Plasmids

XETL plasmid, which carries firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter, was provided by Dr Picard, University of Geneva. S104/106/118A-ER, S118A-ER, and S167A-ER plasmids were mutated in serine residues 104, 106, 118, or 167 to Ala, respectively (a gift from Dr DA Lannigan, University of Virginia); HE241G ERα plasmid mutant that lacks a nuclear translocation signal (NLS) (Δ250-303) (kindly provided by Dr P Chambon, CNRS-INSERM, University of Louis Pasteur). mERα plasmid containing the AF-2/ligand binding domain and a signal that targets this portion of the receptor to the plasma membrane (generously provided by Dr ER Levin, University of California).

III.7 Transfections and luciferase assays

MCF-7 cells were transfected using the FuGENE 6 reagent as recommended by the manufacturer with the mixture containing 0.5 µg of reporter plasmid XETL.

A set of experiments was performed cotransfecting XETL HeLa cells with XETL and HEGO. Another set of experiments was performed by using 0.5 µg/well

pSG5/HE15 (AF-1), pSG5/HE19 (AF-2), HE241G, S104/106/118A-ER, S118A-ER, or S167A-ER plasmids. Six hours after transfection, treatments were added, and cells were incubated for 48 h. A concentration 10 μM, of ICI 182,780 (Tocris Bioscience), Compound C (Enzo Life Sciences), H89 and PP2 (Sigma), PD98059 (Calbiochem), was used. TK Renilla luciferase plasmid (25 ng/well) serves to normalize the efficiency of the transfection. Firefly and Renilla luciferase activities were measured using a Dual Luciferase kit. The firefly luciferase data for each sample were normalized on the basis of transfection efficiency measured by Renilla luciferase activity as reported above (De Amicis F. et al., 2009).

III.8 Immunocytochemical staining

Paraformaldehyde-fixed MCF-7 cells were used for immunocytochemical staining. Endogenous peroxidase activity was inhibited by hydrogen peroxide, and nonspecific sites were blocked by normal horse serum. ERα immunostaining was then performed using as primary antibody a mouse monoclonal antiserum, whereas a biotinylated horse-anti-mouse IgG was utilized as secondary antibody. Avidin-biotin-horseradish peroxidase complex (ABC complex/horseradish peroxidase) was applied, and the chromogen 3,3'-diaminobenzidine tetrachloride dihydrate was used as detection system. TBS-T (0.05 M TRIS- HCl plus 0.15 M NaCl, pH 7.6 containing 0.05% Triton X-100) served as washing buffer. The primary antibody was replaced by normal mouse serum at the same concentration in control experiments on MCF-7 cultured cells.

III.9 Reverse transcription-PCR assay

The gene expression of ERα, cathepsin D, pS2, Cyclin D1 and 36B4 was evaluated by reverse transcription PCR (RT-PCR) method as described (Casaburi I., et al., 2012). Primer sequences include:

estrogen receptor α (ERα), forward 5′-TGATTGGTCTCGTCTGGCG-3′ and reverse 5′-CATGCCCTCTACACATTTTCCC-3′;

Cathepsin D (CatD), forward 5′-AACAACAGGGTGGGCTTC-3′ and reverse 5′-TTTGAGTAGTCAAAGTCAGAGC-3′;

Trefoil factor 1/pS2 (pS2), forward 5′-TTCTATCCTAATACCATCGACG-3′ and reverse 5′-TTTGAGTAGCAAAGTCAGAGC-3′;

Cyclin D1 forward 5'-TCTAAGATGAAGGAGACCATC-3' and reverse 5'-GCGGTAGTAGGACAGGAAGTTGTT-3';

36B4, forward 5′-CTCAACATCTCCCCCTTCTC-3′ and reverse 5′-CAAATCCCAT ATCCTCGT-3′.

Equal amounts of PCR product were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

III.10 *In Vivo* experiments

Female 45-day-old athymic nude mice (*nu/nu Swiss*; Harlan Laboratories Milan, Italy) were maintained in a sterile environment. MDA-MB-231 and MCF-7 cells were pretreated with or without 1 and 5 μg/ml recombinant human gAdiponectin/gAcrp30 (R&D Systems) for 72 h. At day 0, estradiol pellets (1.7 mg per pellet, 60-day release; Innovative Research of America, Sarasota, FL)

were subcutaneously implanted into the intrascapular region of the mice receiving inoculation of ERα-positive MCF-7 cells. The next day, 5 x 10^6 cells were inoculated subcutaneously in 0.1 ml of Matrigel (BD Biosciences, Bedford, MA). Xenograft tumor growth was monitored twice a week by caliper measurements, and tumor volumes (in cubic centimeters) were estimated by the following formula: TV = a x (b^2)/2, where a and b are tumor length and width, respectively, in centimeters. At day 35, the animals were sacrificed following standard protocols; the tumors were dissected from the neighboring connective tissue, frozen in nitrogen, and stored at -80°C for further analyses. All the procedures involving animals and their care were conducted in accordance with the institutional guidelines and regulations at the University of Calabria, Italy. The project was approved by the local ethical committee.

III.11 Histologic Analysis

Formalin-fixed, paraffin-embedded sections of tumor xenografts were sectioned at 5 μm, and stained with hematoxylin and eosin Y (Bio-Optica, Milan, Italy). The epithelial nature of the tumors was verified by immunostaining with mouse monoclonal antibody directed against human cytokeratin 18 (Santa Cruz Biotechnology, Milan, Italy), and nuclei were counterstained with hematoxylin. For negative controls, non-immune serum replaced the primary antibody.

III.12 Statistical analysis

Each datum point represents the mean ± S.D. of at least three independent experiments. Data were analyzed by Student's t test using the GraphPad Prism 4 software program. Statistical comparisons for in *vivo* studies were made using the Wilcoxon-Mann-Whitney test. $P<0.05$ was considered as statistically significant.

RESULTS

IV. 1 Adiponectin induces divergent effects on breast cancer cell proliferation

We first investigated the effect of adiponectin (1 and 5 μg/ ml) on cell proliferation in both estrogen receptor α-negative (ERα−) as well as positive (ERα+) breast cancer cell lines, by MTT growth assays. After 3 days of treatment, adiponectin inhibited cell proliferation of ERα− MDA-MB-231 and SKBR-3 cells, whereas it induced growth in MCF-7 and T47D cells (Fig. IV-1A), which express high levels of ERα (Sflomos G. & Brisken C., 2013). The same dichotomic pattern has been reproduced in anchorage-independent growth assays, using both ERα+ and ERα− breast cancer cells (Fig. IV-1B). We then performed three-dimensional MCF-7 cell cultures, which closely mimic some *in vivo* biologic features of tumors (Mauro L. & Surmacz E., 2004). Our results demonstrated that adiponectin treatment enhanced cell-cell adhesion (Fig. IV-1C) as well as increased cell growth (Fig.IV-1D) compared with untreated cells. In order to investigate the role of ERα in modulating the effect of adiponectin on cell proliferation, in MCF-7 cells ERα was knocked down by siRNA or abrogated by the potent and specific antiestrogen ICI 182,780. In these conditions the adiponectin-induced cell proliferation was completely reversed (Fig. IV-1E). These results address how adiponectin may affect breast cancer growth through the involvement of ERα.

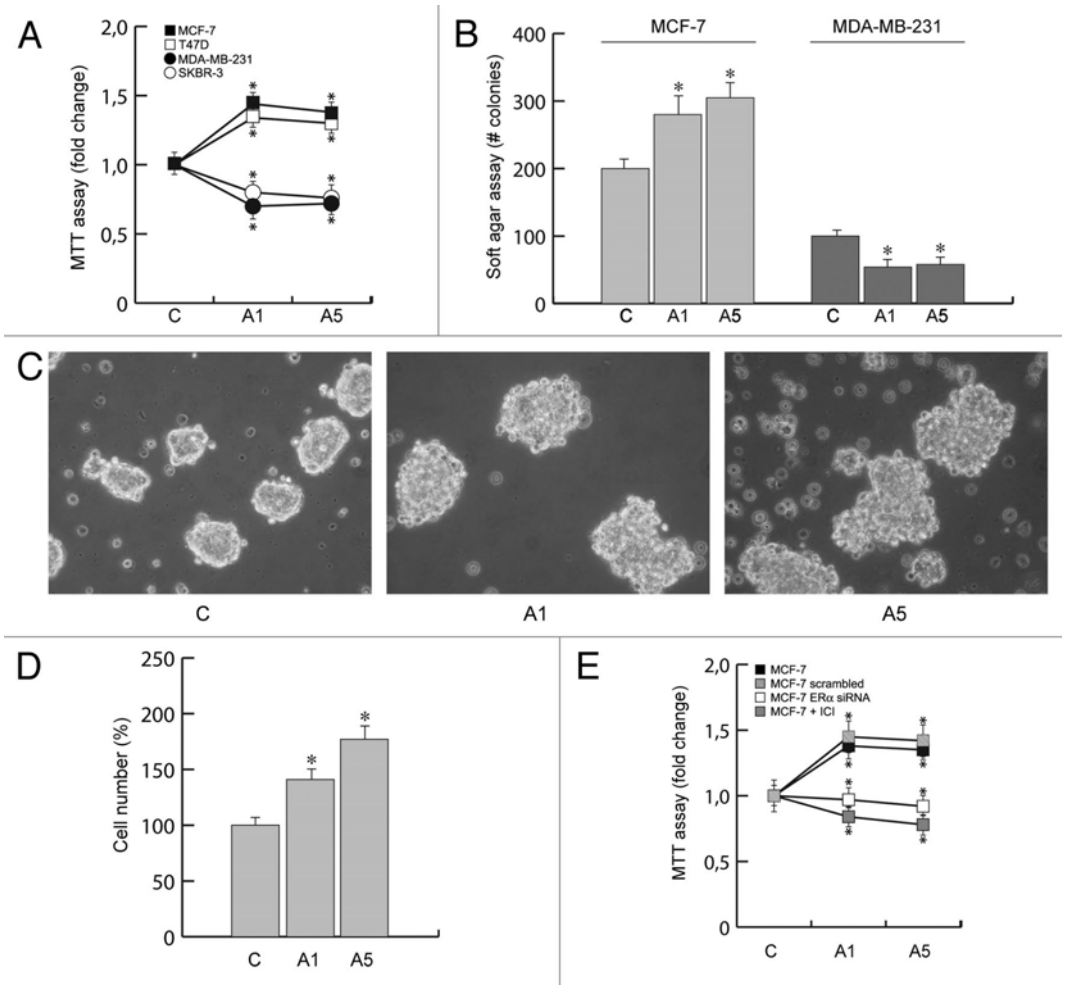

Fig IV-1. Effects of adiponectin on breast cancer cell growth. (A) MTT growth assays in MCF-7, T47D, MDA-MB-231, and SKBR-3 cells treated with vehicle (C) or adiponectin 1 (A1) or 5 µg/ml (A5) for 72 h. Cell proliferation is expressed as fold change ± SD relative to vehicle-treated cells and is representative of 3 different experiments each performed in triplicate. (B) Soft agar growth assay in MCF-7 and MDA-MB-231 cells plated in 0.35% agarose and treated as indicated above. After 14 d of growth colonies >50 µm diameter were counted. (C) MCF-7 three-dimensional cultures were untreated or treated as indicated for 48 h and then photographed under phase-contrast microscopy. (D) Cell numbers obtained from three-dimensional spheroids in MCF-7 cells treated as indicated for 48 h. (E) MCF-7 cells exposed to ICI 182 780, or transfected in suspension with 30 nM siRNAs/well (ERα siRNA or a scrambled siRNA for control samples), were treated with vehicle (C) or A1 or A5 for 72 h before testing cell viability using Mtt assay. Results are expressed as fold change ± SD relative to vehicle-treated cells and are representative of 3 different experiments, each performed in triplicate. *P < 0.05 compared with vehicle.

IV.2 Effect of adiponectin on ERα non-genomic signal

The biological actions of ERα are mediated by non-genomic action outside of the nuclear compartment and by genomic effects via nuclear ERs (Simoncini T. & Genazzani A.R., 2003). The non-genomic effects lead to the rapid activation of the MAPK signaling pathway. Both the genomic and non-genomic actions of ERα play pivotal roles in ERα-induced cancer cell proliferation and survival. Thus, we investigated the possible cross-talk existing between membrane ERα transductional pathways and adiponectin receptor, since both signaling appear to converge to MAPK cascade. Indeed, it has been demonstrated that AdipoR1 binds to the adaptor protein APPL1 (Mao X. et al., 2006) and induces activation of MAPK through Src pathway (Lee M-H., et al., 2008). For instance, c-Src is also an initial and integral crossroad of different membrane signalling events mediated by the ERα as well as by its cross-talk with growth factors. Thus, we found reasonable to verify whether AdipoR1/APPL1 may interact with other membrane signalling involved in breast cancer cell growth and progression. We demonstrated for the first time how, in basal conditions, APPL1 coimmunoprecipitated with AdipoR1, ERα, IGF-IR, and c-Src, but not with EGF-R at both 15 min (Fig. IV-2A) and 48 h (Fig. IV-2B). These interactions appeared enhanced by adiponectin exposure (Fig. IV-2A e 2B). Figure IV-2C shows the *in vitro* effect of the protein complex on Src kinase activity. MCF-7 cell lysates were immunoprecipitated with an anti-APPL1 antibody, and the Src kinase activity was measured using the exogenous acid-treated enolase as substrate in the absence and in the presence of adiponectin after 15 min of exposure. The results provide evidence that adiponectin activates c-Src, as evidenced by both the autophosphorylation of c-Src and the concomitant

phosphorylation of enolase. Furthermore, we observed an increase of IGF-IR phosphorylation after 15 and 30 min of adiponectin treatment, which was reduced after knockdown of ERα (Fig. IV-2D). In MCF-7 cells we observed a rapid activation of MAPK by adiponectin (Fig. IV-2E). To determine the mechanism through which AdipoR1 stimulates MAPK, we employed a panel of RNA silencers targeting ERα, IGF-IR, and APPL1 (Fig. IV-2F), or selective inhibitor targeting Src family kinase or PKA. We observed that MAPK activity was abrogated in the presence of all these agents (Fig. IV-2E), suggesting that MAPK cascade upon adiponectin exposure requires APPL1, ERα, IGF-IR, c-Src, and PKA activity.

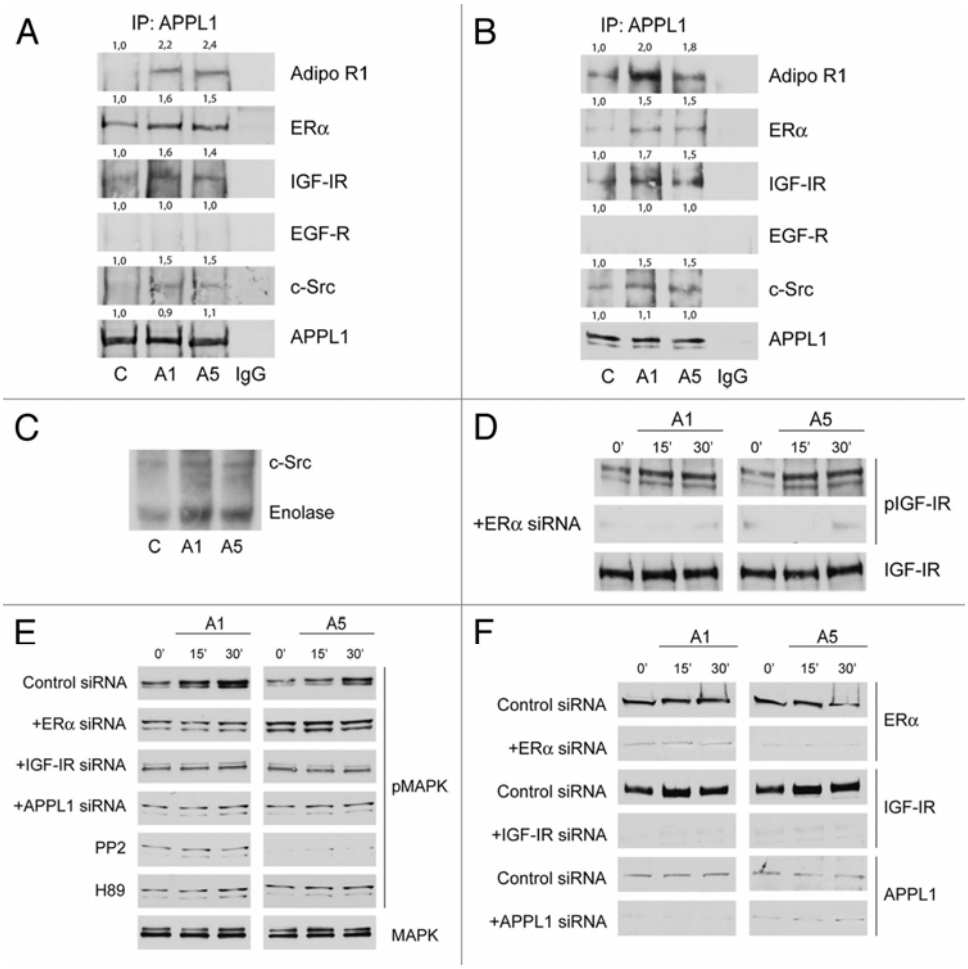

Fig IV-2. Adiponectin induces MAPK activation through the formation of a multimeric protein complex. Five hundred micrograms of protein lysates from MCF-7 cells, untreated (C) or treated with A1 or A5 for 15 min (A) or 48 h (B), were immunoprecipitated with an APPL1 antibody and then blotted with the indicated antibodies on the right. To verify equal loading, the membrane was probed with anti-APPL1 antibody. Normal rabbit IgG was used as a negative control to precipitate A5-treated samples. Numbers on top of the blots represent the average fold change vs. untreated cells. One of three similar experiments is presented. (C) MCF-7 cells, treated with adiponectin for 15 min, were lysed and immunoprecipitated with an anti-APPL1 antibody/protein A/G complex and assayed for c-Src-kinase activity using acid-treated enolase as described in "Materials and Methods". These results are representative of three independent experiments. c-Src and enolase position is indicated. (D) pIGF-IR^{Tyr1131} levels in MCF-7 cells treated with vehicle (−) or A1 and A5 as reported in absence or presence of ERα siRNA. (E) Total cellular proteins were isolated from MCF-7 cells in the absence or presence of ERα, IGF-IR, or APPL1 siRNA or pre-treated with PP2 or H89 and treated with A1 and A5. pMAPK, levels were evaluated by immunoblotting. Total MAPK was used as a loading control. (F) ERα, IGF-IR, and APPL1 levels were shown as control of silencing.

The role of ERα membrane signal in the above reported membrane complex emerges from the evidence that in ERα− MDA-MB-231 cells, adiponectin was no longer able to induce MAPK phosphorylation (Fig. IV-3A). However, when these cells were transfected with a membrane ERα (mERα) construct, which consists solely of the AF-2/ligand binding domain of ERα, adiponectin short exposure was able to upregulate MAPK activity (Fig. IV-3B).

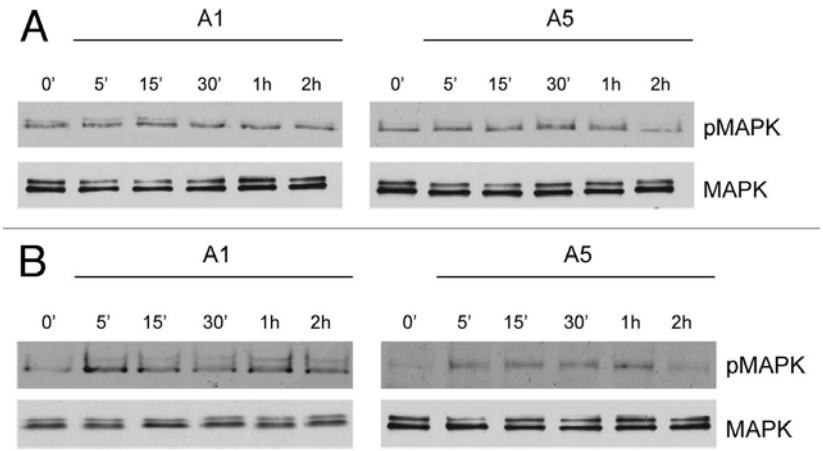

Fig IV-3. Time course of MAPK activation upon adiponectin exposure in MDA-MB-231 cells ectopically expressing membrane ERα. MDA-MB-231 (A) and MDA-MB-231 transfected with a plasmid codifying for membrane ERα (B) were serum-starved for 24 h followed by treatment with adiponectin 1 or 5 μg/ml for the indicated times. Western blots show the phosphorylation status of MAPK. Total MAPK is used as a loading control. One of three similar experiments is presented.

IV.3 Adiponectin transactivates ERα through MAPK signaling

Several studies in recent years suggest that membrane-initiated signaling can converge into genomic events, leading to more long-term consequences. For instance, in MCF-7 breast cancer cells adiponectin revealed an induced MAPK activation still after 48 h treatment (Fig. IV-4A). Since MAPK is involved in enhancing ERα functional activation in a ligand-independent manner, we wondered whether adiponectin was able to modulate ERα transactivation. In MCF-7 cells exposed to adiponectin for 48 h, a significant activation of the estrogen-responsive gene XETL was observed (Fig. IV-4B). Similar results were reproduced in ER-negative HeLa cells ectopically expressing ERα and transfected with XETL (Fig. IV-4B). In the presence of the MAPK inhibitor PD98059, the direct capability of adiponectin in transactivating ERα was completely lost. When we tested different inhibitors of signaling converging on MAPK activation, we observed how PKA and c-Src inhibitors, H89 and PP2 respectively, were able to prevent adiponectin-induced ERα transactivation (Fig. IV.4C). Moreover, knocking down of IGF-IR exerted similar effects (Fig. IV.4D). These results address MAPK signaling as crucial in modulating ERα transactivation upon adiponectin exposure. In addition, we also studied which functional domain was involved in ERα transactivation by adiponectin.

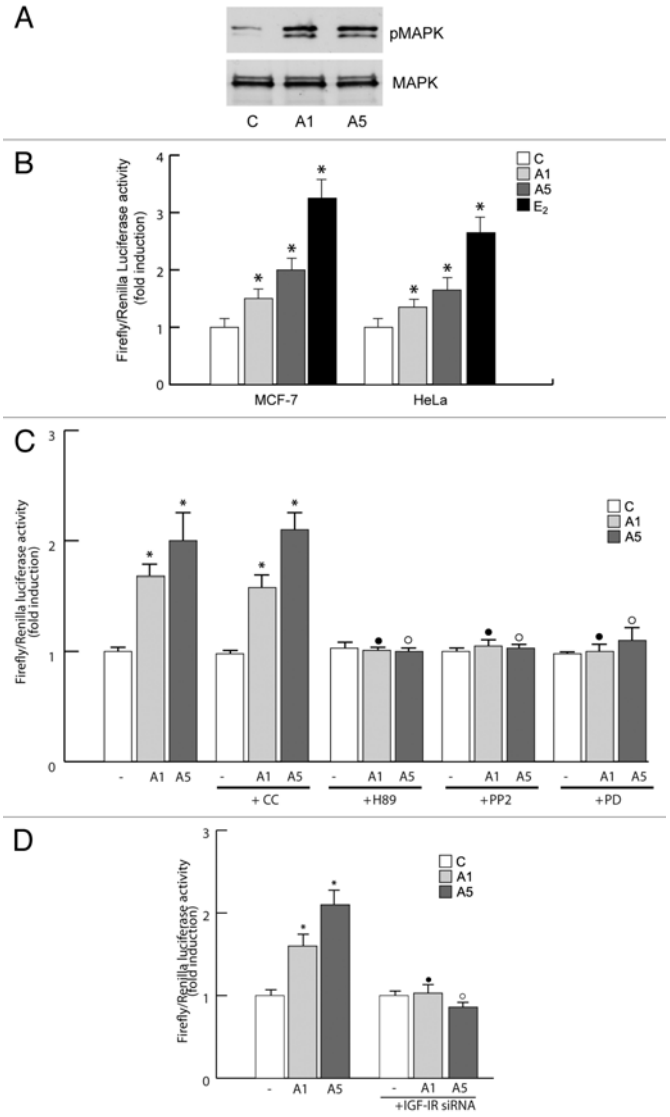

Fig IV-4. Effects of adiponectin on ERα transactivation. (A) Total cellular proteins were isolated from MCF-7 cells treated with A1 and A5 for 48 h. pMAPK, levels were evaluated by immunoblotting. Total MAPK was used as a loading control. (B) MCF-7 cells were transfected with the luciferase reporter plasmid XETL. HeLa cells were cotransfected with XETL and HEGO plasmids. The cells were untreated or treated for 48 h with A1 and A5 or 100 nM E_2, used as positive control. (C) MCF-7 cells were transfected with the luciferase reporter plasmid XETL. The cells were untreated or treated for 48 h with A1 and A5 or in combination with Compound C (CC), H89, PP2, or PD98059. *P < 0.05 compared with control (−); •P < 0.05 compared with A1; ○P < 0.05 compared with A5. (D) MCF-7 cells were transfected with XETL plasmid in the absence or presence of IGF-IR siRNA and treated with adiponectin for 48 h. *P < 0.05 compared with control (−). The values represent the means ± SD of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections.

To this aim, HeLa cells were cotransfected with the XETL reporter gene and plasmids codifying for AF-1 or AF-2 domain (Fig. IV-5A). The treatment with adiponectin induced an increased transcriptional activation only in transfected cells bearing the plasmid codifying for AF-1 domain (Fig. IV-5B). These data demonstrate that the N-terminal AF-1 domain is essential in mediating the adiponectin response. Moreover, the activation of ERα occurred at the genomic level, as demonstrated by trasfecting HeLa cells with the plasmid HE241G, encoding ERα lacking the nuclear localization sequence (Fig. IV-5B). The involvement of AF-1 domain in the activation of ERα by adiponectin was confirmed cotransfecting HeLa cells with XETL and with either HEGO or different plasmids in which ERα was mutated in specific phosphorylation sites, such as Ser-104/106/118A-ER or S118A-ER or S167A-ER (Fig. IV-5C), which are effectors of phosphorylative signaling. As shown in figure IV-5D, in transfectants with either Ser-104/106/118A-ER or S118 mutants, adiponectin was no longer able to elicit any substantial activation on ERE luciferase signal as compared with the cells bearing wild-type ERα. In contrast, the activation still persisted in cells transfected with S167 mutant (Fig. IV-5D). Western blotting analysis confirmed how adiponectin enhanced the phosphorylation of ERα at Ser118, while it was unable to affect phosphorylation at Ser167 (Fig. IV-5E). The biological correlates of these events reproduce the classic features of ERα transactivation in breast cancer cells.

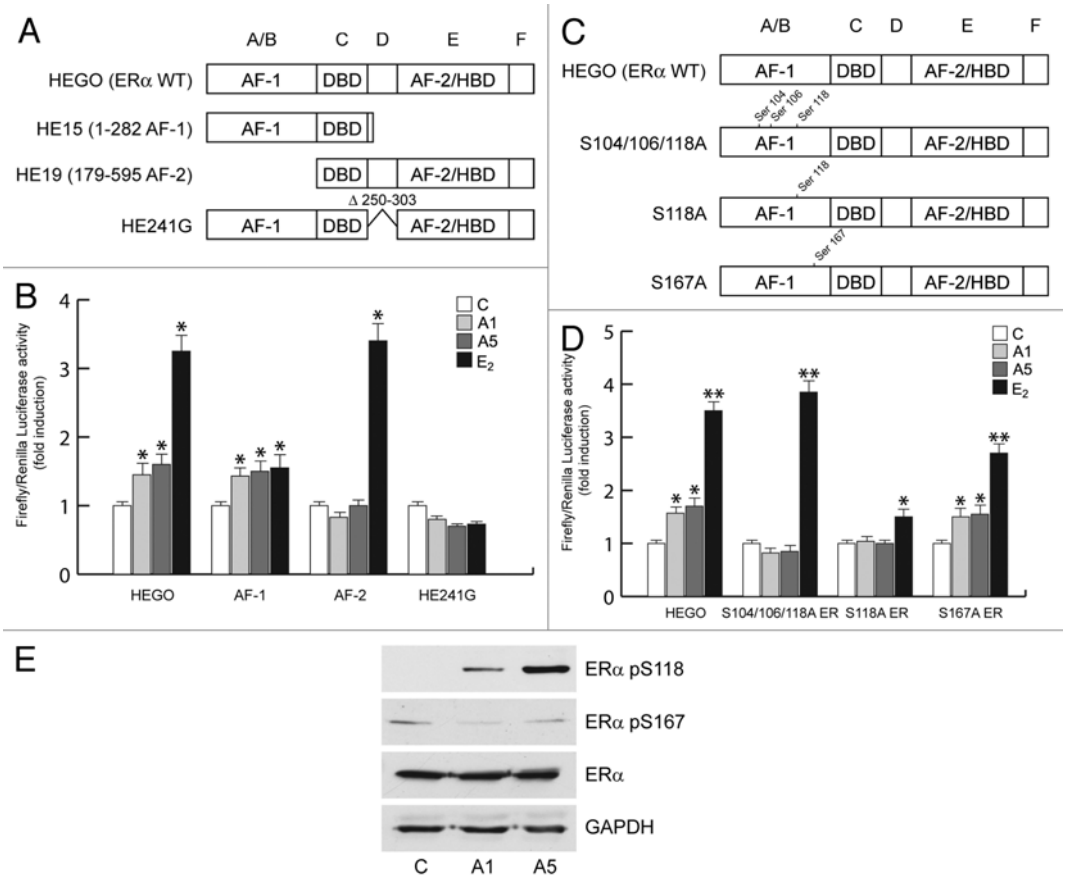

Fig IV-5. Effects of adiponectin on ERα functional domains. (A and C) Schematic illustration of ER constructs used for the experiments. HEGO is a wild-type ER-expressing vector that encodes a 595-amino acid protein. HE15-(1-282) contains AF-1 and the DNA binding domain (DBD). HE19-(179-95) contains DBD, and AF-2 domains. HE241G encodes a mutated ERα, which has the nuclear localization sequence deleted (250-303). ER plasmids mutated in serine residues 104, 106, 118, and 167 to Ala. (B) HeLa cells were transiently cotransfected with XETL and either HEGO or PSG5/HE15 (AF-1) or PSG5/HE19 (AF-2) or HE241G plasmids. (D) HeLa cells were transiently cotransfected with XETL and either HEGO or Ser-104/106/118A-ER or Ser-118A-ER or Ser-167A-ER. The cells were treated for 48 h in the absence (C) or in the presence of A1 and A5 or E_2 (100 nM) used as positive control. The values represent the means ± SD of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. **P* < 0.05, ***P* < 0.01 compared with (C). (E) Total extracts from cells treated with A1 or A5 for 30 min were analyzed for phosphorylation of serines 118 and 167 (pS118 and pS167) and expression of ERα by immunoblot analysis. GAPDH was used as a control for equal loading and transfer.

In MCF-7 cells cultured in serum deprivation conditions for 96 h, ERα immunoreactivity was no longer detectable in the control, whereas treatment with adiponectin for 24h induced a strong ERα immunoreactivity in the nuclear compartment (Fig. IV-6A). These results show that adiponectin mimics the effect of estradiol on ERα compartmentalization in breast cancer cells. In addition, in MCF-7 cells, we observed a significant downregulation of ERα mRNA and protein levels after adiponectin treatment (Fig. IV-6B and C), which is a typical hallmark of the receptor activation by an agonist. Moreover, adiponectin was able to increase the expression of classic estrogen-dependent genes, such as Catepsin D and pS2 (Fig. IV-6D), providing further evidence for the ability of this adipokine to transactivate ERα.

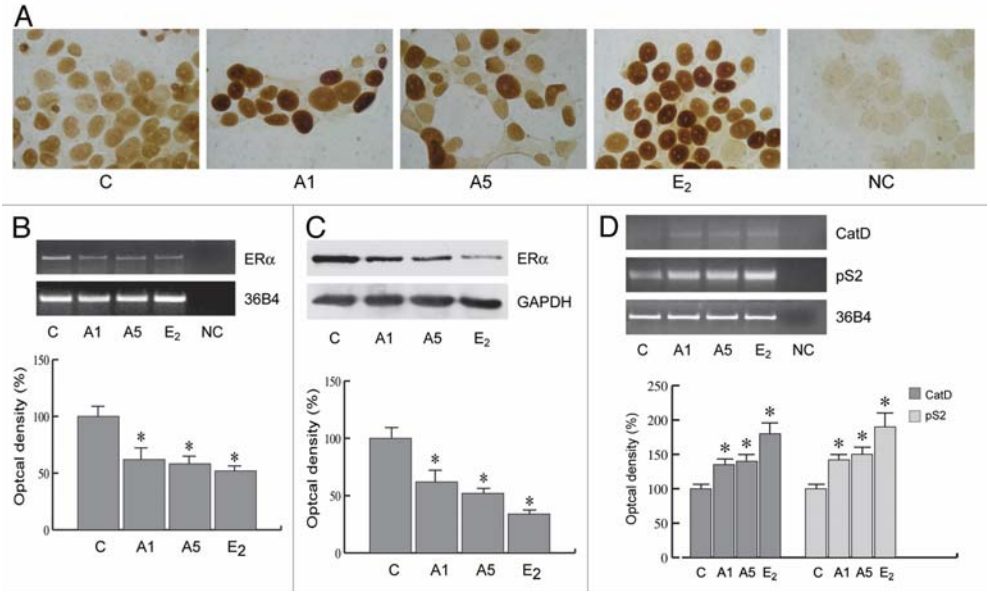

Fig IV-6. Adiponectin actions on cellular localization and expression of ERα. (A) MCF-7 cells were incubated in serum-free medium for 96 h and then treated with vehicle (C), A1, A5, or 100 nM E_2, used as positive control, for 24 h. No immunodetection was observed replacing the anti-ER antibody with an irrelevant mouse IgG (NC). Each experiment is representative of at least 10 tests. (B) RT-PCR of ERα mRNA. MCF-7 cells were stimulated for 48 h with A1 and A5 or E_2 (100 nM); 36B4 mRNA levels were determined as a control. (C) Immunoblot of ERα from MCF-7 cells treated as above; GAPDH serves asloading control. (D) RT-PCR of Catepsin D (CatD) and pS2 mRNA. MCF-7 cells were treated as reported. The histograms represent the mean ± SD of three separate experiments, in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *P<0.05 vs. control.

IV.4 Effects of adiponectin on breast cancer tumor cell growth *in vivo*

Finally, we evaluated the effects of adiponectin on the development of breast carcinomas in nude mouse models. MDA-MB-231 and MCF-7 cells were pretreated with or without adiponectin (1 and 5 μg/ ml) for 72 h and then injected into the intrascapular region. All the *in vivo* procedures were well tolerated because no change in body weight or in food and water consumption was observed together with no evidence of reduced motor function. In addition, no significant difference in the mean weights or histologic features of the major organs (liver, lung, spleen, and kidney) after sacrifice was observed between vehicle-treated mice and those that received pre-treated cells. The *in vivo* data showed that on day 35 adiponectin treatment induced a significant reduction (60 and 40% at doses of 1 and 5μg/ml, respectively) in tumor volume in mice injected with MDA-MB-231 cells (Fig. IV-7A), whereas an increased tumor growth (54 and 109% at doses of 1 and 5μg/ml, respectively) was observed in the animal groups receiving pretreated MCF-7 cells (Fig. IV-7B).

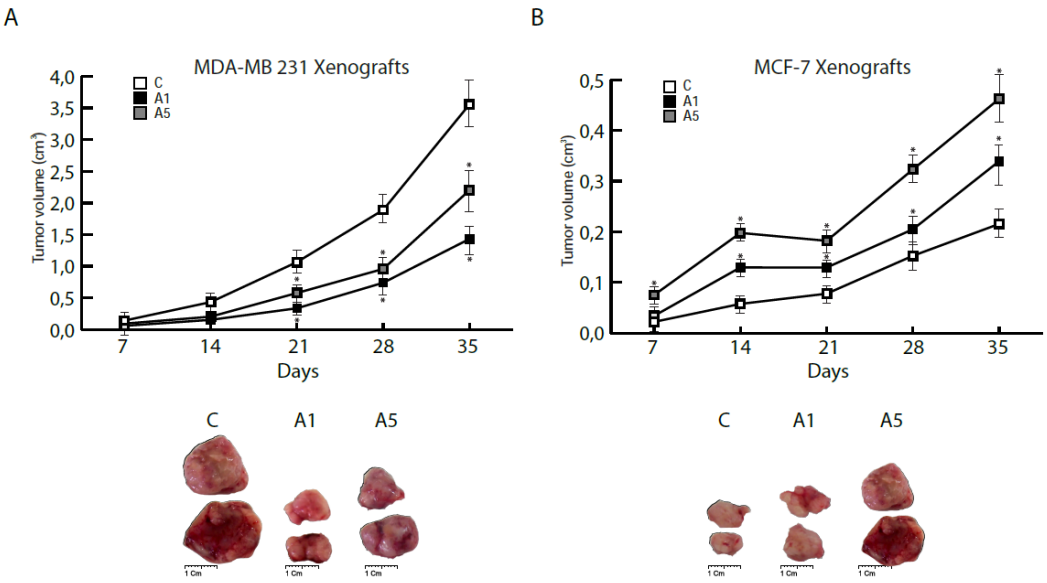

Fig IV-7. Effects of adiponectin on the growth of breast cancer cell-derived tumor in nude mice. MDA-MB-231 (**A**) and MCF-7 (**B**) cells were untreated or pretreated with adiponectin 1 (A1) or 5 (A5) µg/ml for 72 h and then implanted into the intrascapular region of female nude mice. Tumor growth was monitored by measuring the visible tumor sizes at various time points. Representative images of MDA-MB-231 and MCF-7 xenograft tumors collected at 35th days are shown. n = 8 mice per group, from three independent studies. *p<0.05 versus control group.

To distinguish the xenograft from the mouse tissue we performed immunostaining on xenograft sections with hematoxylin and eosin. As evidenced in the Supplemental Figure 1A, histological examination of MDA-MB-231 and MCF-7 xenografts revealed that tumors were primarily composed of tumor epithelial cells. Moreover, the epithelial nature of the tumors was verified by immunostaining with antibody direct against human cytokeratin 18 (Fig. IV-8).

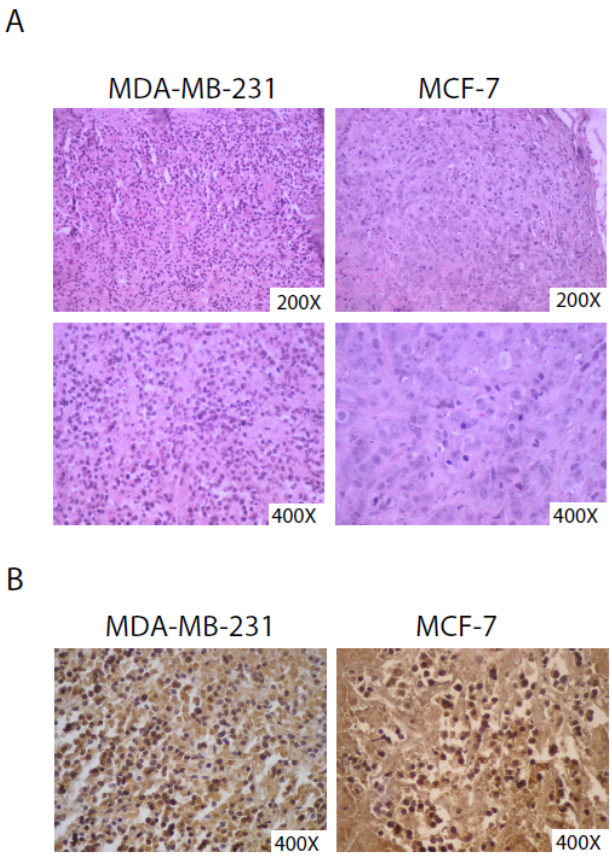

Fig IV-8. Staining of MDA-MB-231 and MCF-7 xenograft tumors. Representative tumor sections from mice at 35th days were formalin fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin Y (A) or incubated with a mouse monoclonal antibody directed against human cytokeratin 18 (B). Cytokeratin 18 expression appears as brown cytoplasmic staining.

To determine whether the effects in tumor growth induced by adiponectin were associated with any changes in the mitotic index, we evaluated in tumors the expression of Ki-67 as a marker of proliferation. Sections of tumors from adiponectin-treated mice exhibited a reduced expression of Ki-67 in MDA-MB-231 cells (Fig.IV-9A), while increased levels were observed in MCF-7-derived tumors compared with that in tumors from control mice (Fig. IV-9B).

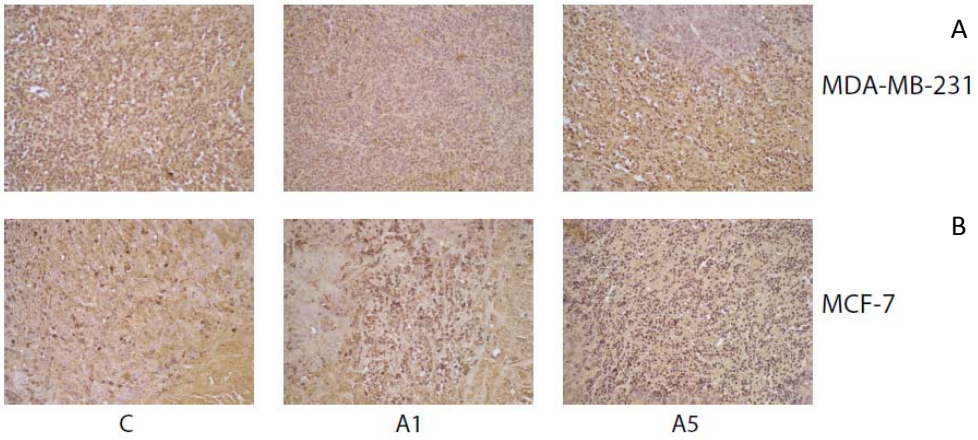

Fig IV-9. Staining of Ki67 in xenograft tumors. Representative tumor sections at 35th days from mice injected with MDA-MB-231 (A) or MCF-7 (B) cells were formalin fixed, paraffin embedded, sectioned, and incubated with a mouse monoclonal antibody directed against Ki67.

The binding of adiponectin to its receptors provokes the activation of AMPK via APPL1 which is considered a cellular energy sensor that is stimulated by an increase of intracellular AMP/ATP ratio (Obeid S. & Hebbard L., 2012). On the other hand the proliferative effect could be also explained by the activation of ERK1/2 MAPK which is crucial for cell cycle initiation, cell growth and survival (Dalamaga M. et al., 2012).

Focusing our attention on the above reported signaling, it was extremely interesting to observe how in protein extracts obtained from mice xenografted with MDA-MB-231 cells pretreatment with adiponectin we evidenced an enhanced activation of AMPK signaling (Fig. IV-10A). In contrast, in MCF-7 tumor xenografts we observed an increased MAPK phosphorylation concomitant with a progressive decrease of AMPK (Fig. IV-10B).

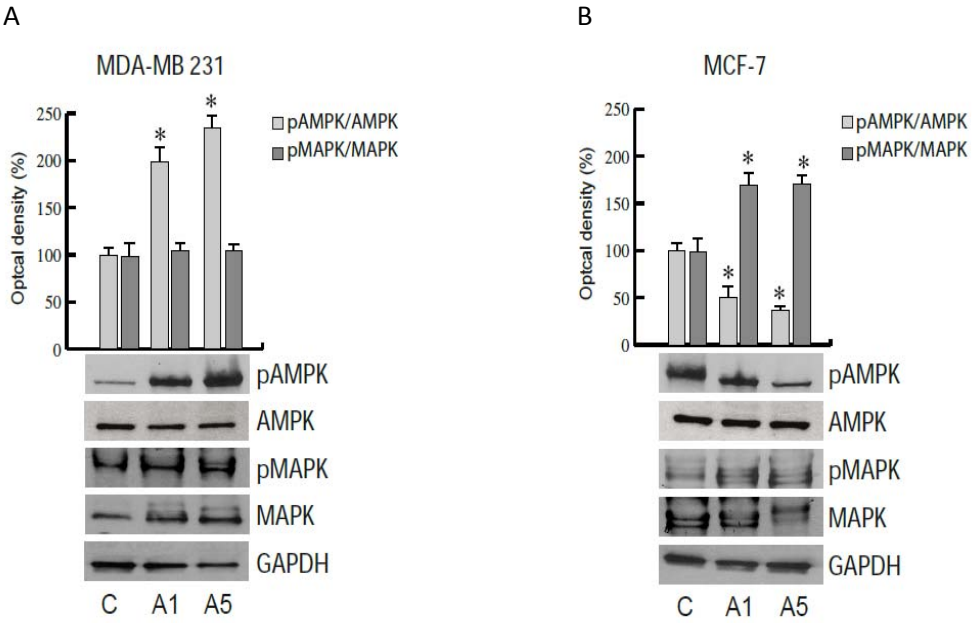

Fig IV-10. Representative Western blot analysis on protein extracts from MDA-MB-231 (A) or MCF-7 (B) xenograft tumors excised from mice showing AMPK and MAPK activation. The immunoblots were stripped and reprobed with total AMPK and MAPK. GAPDH was used as loading control. The results are mean ± S.D. of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed to be 100%. *$p<0.05$ versus untreated cells.

Our *in vivo* results confirmed the divergent effects of adiponectin on breast cancer cell growth in relation to the ERα expression as we previously demonstrated in *in vitro* studies (Mauro L. et al., 2014).

IV.5 Modulatory role of adiponectin on cyclin D1 expression

Stimulation of cell growth is accompanied by the coordinate expression of multiple genes and pathways including those required for different phases of cell cycle progression (Fu M . et al., 2004). It is well known that ERα drives breast cancer cell proliferation through the regulation of the expression levels of cyclin D1, that controls the G1-S phase transition of the cell cycle (Foster, J. S. et al., 2001).

Thus, we investigated whether adiponectin may modulate cyclin D1 expression. *In vivo* adiponectin triggered opposite effects on cyclin D1 expression at both mRNA and protein level: a downregulation in MDA-MB-231-derived tumors (Fig. IV-11A) and an upregulation in MCF-7 xenografts (Fig. IV-11B). Similar results were obtained *in vitro* using MDA-MB-231 (Fig. IV-11C) and MCF-7 (Fig. IV-11D) cells treated with adiponectin for 48 h.

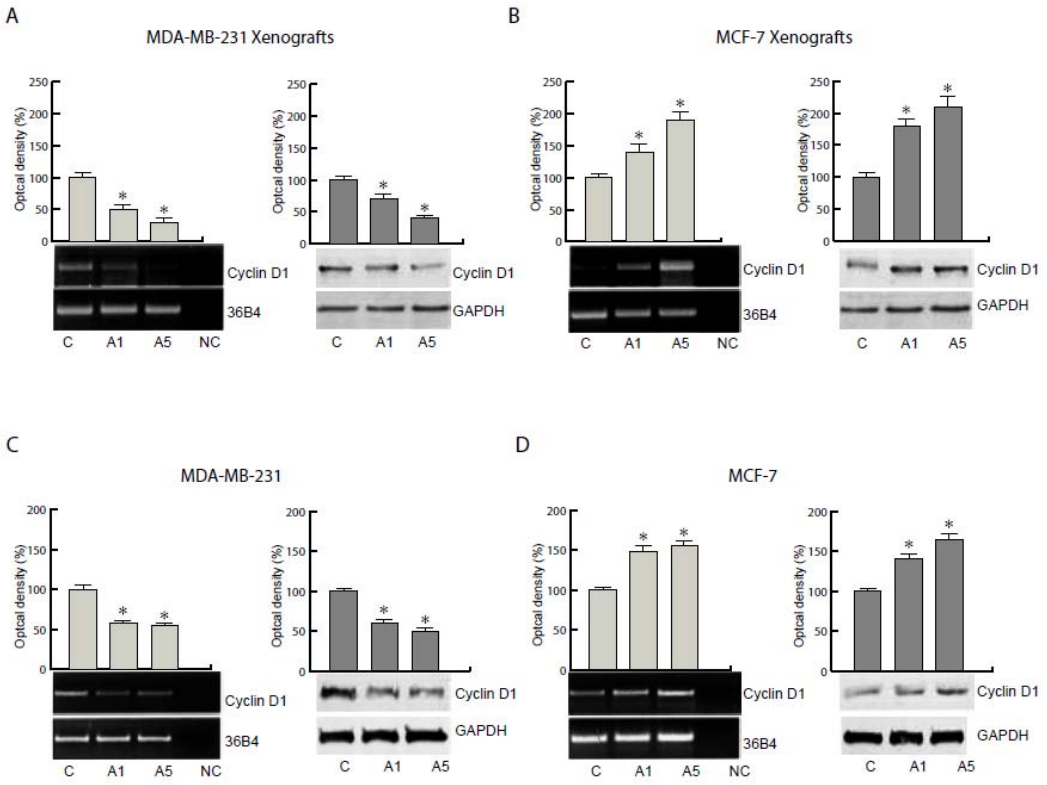

Fig IV-11. Effects of adiponectin on cyclin D1 expression. mRNA and protein levels of cyclin D1 were done in MDA-MB-231 (A) and MCF-7 (B) tumor xenografts. MDA-MB-231 (C) and MCF-7 (D) cells were starved and treated with adiponectin 1 or 5 µg/ml for 48 h or left untreated. Total RNA was reverse transcribed and cDNA was subjected to PCR using specific primers for cyclin D1 or 36B4. NC: negative control, RNA sample without the addition of reverse transcriptase. 36B4 mRNA levels were determined as control. Protein extracts were resolved by SDS-PAGE and subjected to immunoblot analysis with rabbit antiserum against human cyclin D1. GAPDH was used as a control for equal loading and transfer. The histograms represent the mean ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *$p < 0.05$ compared to untreated cells.

DISCUSSION

Discussion

Adipose tissue is now widely considered to be an active endocrine organ, secreting several bioactive adipokines, including adiponectin, that exert distinct metabolic functions (Kershaw E.E. & Flier J.S., 2004). Adiponectin plays a protective function against obesity-related disorders and the metabolic syndrome, particularly in the pathogenesis of type II diabetes and cardiovascular disease (Trujillo M.E. & Scherer P.E., 2005). Beyond these metabolic effects, adiponectin deficiency, commonly observed in obesity, may contribute to the natural history of several malignancies, such as breast cancer (Mantzoros C. et al., 2004). In particular, low adiponectin levels have been strongly associated with an increased breast cancer risk, and patients with reduced adiponectin levels develop a biologically aggressive phenotype independently of estrogen receptor status, (Kelesidis I. et al., 2006) although inconclusive results have been published in the latter concern. For instance, several data point toward cell line-dependent effects (Jardé T. et al., 2011). Adiponectin has been shown to inhibit the growth of ERα-negative normal MCF-10A human mammary epithelial cells, (Treeck O. et al., 2008) as well as cancerous MDA-MB-231 (Kang JH. Et al., 2005) and SKBR-3 (Grossmann M.E. et al., 2008) cells. This anti-proliferative effect involves an enhanced expression of Bax, Bcl-2, p53, c-myc, and reduced level of cyclin D1 (Chen X. & Wang Y., 2011).

However, in ERα-positive MCF-7 and T47D cells, adiponectin appears to stimulate (Pfeiler G.H. et al., 2006) or to inhibit cell growth, (Grossmann M.E. et al., 2008) or to play no noticeable effect (Kang J.H. et al., 2005). Possible explanations of the different cellular responses may be different content of ERα, differences in culture

conditions, specific adiponectin isoform used, incubation time, or dosage. Here, we elucidated the complex mechanisms involved in adiponectin response in breast cancer growth in dependency on ERα status. Particularly, we demonstrated that MAPK activation, induced by adiponectin/ERα-mediated effect, produces MCF-7 cell proliferation, and it represents the discriminator factor determining the opposite effect induced by adiponectin in ERα-positive and ERα-negative breast cancer cells. For instance, adiponectin inhibits the growth of ERα-negative breast cancer cells, whereas it induces proliferation in ERα-positive cells. The evidence that this dichotomic effect is dependent on the expression of ERα emerges from the observation that in the presence of its knocking down, adiponectin induced inhibition of MCF-7 cell growth. Thus, we asked through which molecular mechanisms ERα may interfere with adiponectin effect on breast cancer cell growth. It has been described that a fraction of ERα is extranuclear and exists as cytoplasmatic/membrane-bound population that mediates rapid "non-genomic" events, leading to cell morphologic changes and inhibition of apoptosis (Lanzino M. et al., 2008). According to this function, cytoplasmatic ERα can physically interact with different signaling molecules, including IGF-IR (Lanzino M. et al., 2008) and c-Src (Song R.X. et al., 2010). Recent findings demonstrated that AdipoR1, through its most important adaptor protein APPL1, activated c-Src/MAPK pathway as specific effector of adiponectin signaling (Lee M-H. et al., 2008). Thus, APPL1 appears to be a key intracellular mediator of most of adiponectin responses reported to date. Activation of MAPK cascade is essential for cell cycle initiation and plays a key role in the control of cell proliferation, differentiation, and survival, (Pearson G. et al., 2001) hypothesizing that adiponectin might act as a

growth factor. MAPK serves as a point of convergence for diverse membrane signal inputs. All this has given the rationale to ascertain the existence of a mechanistic link existing between AdipoR1/APPL1 and other ERα driven membrane signaling. To date, nearly 14 proteins have been reported to associate with APPL1 in various types of cells, and they could be categorized into 3 different groups: membrane receptors, signaling proteins, and others (Deepa S.S. & Dong L.Q., 2009). On the basis of coimmunoprecipitation assays, we demonstrated that AdipoR1 and APPL1 interact with ERα, c-Src, and IGF-IR, all converging into MAPK activation. Indeed, the latter event was completely abrogated in the presence of ERα, IGF-IR, and APPL1 siRNA or pharmacological inhibitor of c-Src, PP2. The role of c-Src in maintaining protein complex formation is sustained by evidence demonstrating that, in MCF-7 cells, it allows ERα interaction with IGF-IR, exhibiting a docking site for ERα membrane association (Lanzino M. et al., 2008). Moreover, our results demonstrated that short exposure to adiponectin was able to rapidly enhance tyrosine phosphorylation of IGF-IR, which was no longer noticeable in the presence of ERα siRNA. On the basis of these findings, we hypothesize that adiponectin induces a linear pathway involving AdipoR1, ERα/IGF-IR/c-Src/MAPK. The role of ERα in mediating the adiponectin-induced activation of MAPK in breast cancer cells emerges from the evidence that ectopical expression of membrane ERα in MDA-MB-231 cells was able to restore MAPK phosphorylation by adiponectin. This addresses how the presence of rapid ERα membrane signal is a prerequisite for adiponectin-induced MAPK activation. In addition, membrane ERα, driving membrane signaling, may, in the long-term, induce a cascade of events converging in MAPK activation, which, in turn,

transactivates ERα at genomic level. In our study, we have provided the first evidence that adiponectin induces transactivation of ERα in MCF-7 cells. Indeed, it is worth observing that this occurred according to the classical features of ERα transactivation: nuclear localization, downregulation of mRNA and protein levels, and upregulation of estrogen-dependent genes. Adiponectin was able to transactivate ERα in a ligand- independent manner, since only the AF-1 domain is essential in mediating the specific response through the phosphorylation of Ser118. Serine118 in the N-terminus of human ERα is a well-studied phosphorylation site, and both rapid estrogens action and growth factors such as EGF and IGF-I, all converging in MAPK activation, result in phosphorylation of Ser118 (Murphy L.C. et al., 2006). Indeed, our results demonstrated that upon adiponectin exposure, ERα transactivation was abrogated in the presence of MAPK inhibitor PD98059. Also, PKA appears to play a role in the latter concern, since its inhibition abolishes adiponectin-stimulated MAPK activation. In our model, we demonstrated that adiponectin via PKA signal influences MAPK pathway, thus inducing ERα transactivation. Indeed, both adiponectin-induced MAPK phosphorylation and ERα transactivation are completely abrogated in the presence of a specific PKA inhibitor, H89. However, our results showed that adiponectin action on ERα transactivation was independent by phosphorylation of Ser167, targeted by PKA signaling, while it failed to induce ERα signaling in cells ectopically expressing ERα mutated in Ser118, usually phosphorylated by MAPK. All these observations lead to the idea that adiponectin effects on ERα transactivation are based mostly on MAPK signaling, which is dependent on the formation of a multiprotein complex including AdipoR1/APPL1/c-Src/ ERα/IGF-

IR. On the other hand, the activation of MAPK by PKA may occur through c-Src/ Rap1/B-Raf cascade (Luttrell L.M., 2003). Thus the adiponectin/AdipoR1/ERα axis, through c-Src, may converge on MAPK activation. Adiponectin-induced transactivation of ERα appears not to involve another important effector signaling, such as AMPK, since it is not substantially affected by its pharmacological inhibition.

Our *in vitro* studies showed that adiponectin stimulates growth in ERα-positive MCF-7 cells while inhibits proliferation of ERα-negative MDA-MB-231 cells (Mauro L. et al., 2014). These *in vitro* data are further corroborated by our animal study demonstrating that adiponectin could induce or inhibit the breast tumor development in nude mice in relationship to ERα status. In addition, our results evidenced, in sections from xenograft tumors, the same dichotomic pattern in the expression of the nuclear protein Ki67, correlated with proliferation markers (Patani N. et al., 2013).

Among the molecules involved in cell proliferation, cyclin D1 is a critical modulator in the cell cycle G1/S transition (Roy P.G. & Thompson A.M., 2006). Our results demonstrated that in our experimental model the expression of mRNA and protein content of cyclin D1 was decreased in MDA-MB-231 cells and increased in MCF-7 cells by adiponectin, as evidenced by both *in vivo* and *in vitro* results.

In conclusion, we provided evidence that the effects of adiponectin on breast cancer cell proliferation are dependent on ERα expression through both its non-genomic and genomic actions. Thus, our results suggest that ERα negatively interferes with the antiproliferative effect induced by adiponectin on breast cancer cell growth. On the basis of these observations, it is rational to conclude that at the

doses tested, adiponectin exerts an antiproliferative role only in ERα-negative breast cancer cells, wherein it may represent a promising pharmacological tool to be implemented in the novel adjuvant strategies adopted in breast cancer.

REFERENCES

References

1. Abrahamson, P.E., Gammon, M. D., Lund, M. J., Flagg, E. W., Porter, P. L., Stevens, J., Swanson, C. A., Brinton, L. A., Eley, J. W. and Coates, R. J. (2006). General and abdominal obesity and survival among young women with breast cancer. Cancer Epidemiol. Biomarkers Prev., 15(10), 1871-1877.

2. Andersen TI, Heimdal KR, Skrede M, Tveit K, Berg K, Borresen A. (1994). Oestrogen receptor (ESR) polymorphisms and breast cancer susceptibility. Hum Genet; 94: 665-670.

3. Andò S, Catalano S.(2012). The multifactorial role of leptin in driving the breast cancer microenvironment. Nat Rev Endocrinol 8:263-75

4. Argani, P., & Cimino-Mathews, A. (2012). Breast cancer and breast pathology. Accessed October 6, 201

5. Berrino, F., Muti, P., Micheli, A., Bolelli, G., Krogh, V., Sciajno, R., Pisani, P., Panico, S. and Secreto, G. (1996). Serum sex hormone levels after menopause and subsequent breast cancer. J. Natl. Cancer Inst., 88(5), 291-296.

6. Bjørbaek C., Uotani S., Da Silvia B., Flier J. S., (1997). Divergent Signaling Capacities of the Long and Short Isoforms of the Leptin Receptor. The Journal of Biological Chemistry. 272:32686-32695.

7. Brochu-Gaudreau K, Rehfeldt C, Blouin R, Bordignon V, Murphy BD, Palin MF.(2010). Adiponectin action from head to toe. Endocrine 37:11- 32

8. Brodie, A., Lu, Q. and Nakamura, J. (1997). Aromatase in the normal breast and breast cancer. J. Steroid Biochem. Mol. Biol., 61(3-6), 281-286.

9. Bulun, S.E., Mahendroo, M. S. and Simpson, E. R. (1994). Aromatase gene expression in adipose tissue: Relationship to breast cancer. J. Steroid Biochem. Mol. Biol., 49(4-6), 319-326.

10. Calle, E.E., Rodriguez, C., Walker-Thurmond, K. and Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N. Engl. J. Med., 348(17), 1625-1638.

11. Cannon, B. and J. Nedergaard (2004). Brown adipose tissue: function and physiological significance. Physiol Rev, 84(1): p. 277-359.

12. Casaburi I, Avena P, Lanzino M, Sisci D, Giordano F, Maris P, Catalano S, Morelli C, Andò S.(2012). Chenodeoxycholic acid through a TGR5-dependent CREB signaling activation enhances cyclin D1 expression and promotes human endometrial cancer cell proliferation. Cell Cycle 11:2699-710

13. Catalano S. et al., (2004). Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha (ERα) in MCF-7 cells. J Biol Chem 279(19):19908-15.

14. Catalano S., Marsico S., Giordano C., Mauro L., Rizza P., Panno M. L., Andò S., (2003). Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. J Biol Chem. 278:28668-76.

15. Chen X, Wang Y. (2001). Adiponectin and breast cancer. Med Oncol 28:1288-95.

16. Chen, S., Itoh, T., Wu, K., Zhou, D. and Yang, C. (2002) Transcriptional regulation of aromatase expression in human breast tissue. J. Steroid Biochem. Mol. Biol., 83(1-5), 93-99.

17. Cleland, W.H., Mendelson, C. R., Simpson, E. R. (1985). Effects of aging and obesity on aromatase activity of human adipose cells. J. Clin. Endocrinol. Metab., 60(1), 174-177.

18. Clementi F. & Fumagalli G., (1999). General and Molecular Pharmacology. UTET.

19. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, Kahn SE.(2003). Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 46:459-69.

20. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P (2003). Overview of the main outcomes in breast-cancer prevention trials. Lancet; 361: 296-300.

21. Dalamaga M, Diakopoulos KN, Mantzoros CS. (2012). The role of adiponectin in cancer: a review of current evidence. Endocr Rev 33:547-94;

22. Dao TL. (1981). The role of ovarian steroid hormones in mammary carcinogenesis. Hormones and Breast Cancer. Cold Spring Harbor; New York: 281-295.

23. De Amicis F, Russo A, Avena P, Santoro M, Vivacqua A, Bonofiglio D, Mauro L, Aquila S, Tramontano D, Fuqua SA, et al.(2013). In vitro mechanism for downregulation of ER-α expression by epigallocatechin gallate in ER+/PR+ human breast cancer cells. Mol Nutr Food Res 57:840- 53.

24. De Amicis F, Zupo S, Panno ML, Malivindi R, Giordano F, Barone I, Mauro L, Fuqua SA, Andò S.(2009). Progesterone receptor B recruits a repressor complex to a half-PRE site of the estrogen receptor alpha gene promoter. Mol Endocrinol 23:454- 65

25. Deepa SS, Dong LQ.(2009). APPL1: role in adiponectin signaling and beyond. Am J Physiol Endocrinol Metab 296:E22-36.

26. Dickson R. B. & Lippman M. E. (1988). Control of human breast cancer by estrogen, growth factors, and oncogenes. Cancer Treat Res. 40: 119-65.

27. Dickson, R.B. &Lippman, M. E. (1986). Hormonal control of human breast cancer cell lines. Cancer Surv., 5(3), 617-624.

28. Dickson, R.B. & Stancel, G. M. (2000). Estrogen receptor-mediated processes in normal and cancer cells. J. Natl. Cancer. Inst. Monogr., (27), 135-145.

29. Dieudonne M.N., Machinal-Quelin F., Serazin-Leroy V., Leneveu M C., Pecquery R., Giudicelli Y., (2002). Leptin mediates a proliferative response in human MCF7 breast cancer cells. Biochem Biophys Res Commun. 293 622- 628.

30. Edwards D. P. (2005). Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol. 67:335-76.

31. Eerola H, Aittomaki K, Asko-Seljavaara S, Nevanlinna H, von Smitten K. (2002). Hereditary breast cancer and handling of patients at risk Scand J Surg.; 91: 280-287.

32. Evans R. M., (1988). The steroid and thyroid hormone receptor superfamily. Science. 240: 889-95.

33. Ewertz M, Duffy SW, Adami HO, Kvale G, Lund E, Meirik O, Mellemgaard A, Soini I, Tulinius H. (1990). Age at first birth, parity and risk of breast cancer: a metaanalysis of 8 studies from the Nordic countries. Int J Cancer; 46:597-603.

34. Fantuzzi, G. and T. Mazzone (2007). Adipose tissue and adipokines in health and disease. Totowa, N.J. Humana Press.

35. Feigelson, H.S. & Henderson, B. E. (1996). Estrogens and breast cancer. Carcinogenesis, 17(11), 2279-2284.

36. Flouriot G, Brand H, Denger S. (2000). Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J; 19:4688-700.

37. Foster, J.S., Henley, D.C., Ahamed, S., Wimalasena, J. (2001) Estrogens and cell-cycle regulation in breast cancer. Trends Endocrinol. Metab. 12, 320-327.

38. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF. (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc Natl Acad Sci U S A; 98:2005- 10

39. Frühbeck G., Gómez-Ambrosi J., Muruzabal F.J., Burrel M.A.,(2001). The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. Am J Physiol. 280: E827-E847.

40. Fu, M., Wang, C., Li, Z., Sakamaki, T., Pestell, R.G. (2004). Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 145, 5439-5447.

41. Fukuhara A., Matsuda M., Nishizawa M., Segawa K., Tanaka M., Kishimoto K., Matsuki Y., Murakami M., Ichisaka T., Murakami H., Watanabe E., Takagi T., Akiyoshi M., Ohtsubo T., Kihara S., Yamashita S., Makishima M., Funahashi T., Yamanaka S., Hiramatsu R., Matsuzawa Y., Shimomura I. (2005). Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science. 307: 426-430.

42. Garofalo C. & Surmacz E. (2006). Leptin and cancer. J Cell Physio. 207(1):12-22.

43. Green S., Walter P., Kumar V., et al. (1986). Human oestrogen receptor cDNA: sequence. Nature; 320: 134-139.

44. Greene G.L., Gilna P., Waterfield M., Baker A., Hort Y., Shine J. (1986). Sequence and expression of human estrogen receptor complementary dna. Science; 231: 1150-1154.

45. Grodin, J.M., Siiteri, P.K. and MacDonald, P.C. (1973). Source of estrogen production in postmenopausal women. J. Clin. Endocrinol. Metab., 36(2), 207-214.

46. Grossmann ME, Nkhata KJ, Mizuno NK, Ray A, Cleary MP. (2008). Effects of adiponectin on breast cancer cell growth and signaling. Br J Cancer; 98:370- 9.

47. Guido C, Panza S, Santoro M, Avena P, Panno ML, Perrotta I, Giordano F, Casaburi I, Catalano S, De Amicis F, et al.(2012). Estrogen receptor beta (ERβ) produces autophagy and necroptosis in human seminoma cell line through the binding of the Sp1 on the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) promoter gene. Cell Cycle; 11(15):2911-21.

48. Guido C, Whitaker-Menezes D, Lin Z, Pestell RG, Howell A, Zimmers TA, Casimiro MC, Aquila S, Ando' S, Martinez-Outschoorn UE, et al.(2012). Mitochondrial fission induces glycolytic reprogramming in cancer-associated myofibroblasts, driving stromal lactate production, and early tumor growth. Oncotarget; 3:798-810

49. Hankinson, S., Tamimi, R., & Hunter, D. (2008). Breast cancer. In H.-O. Adami, D. Hunter, & D. Trichopoulos (Eds.), Textbook of cancer epidemiology (2nd ed. ed., pp. 403-445).

50. Harvie M., Hooper L., Howell A. H., (2003). Central obesity and breast cancer risk: a systemic review. Obesity Reviews. 4 157-173.

51. Helmrich et al. (1983). Risk Factors for breast cancer. Am. J. Epidemiol. 1: 35-45.

52. Henderson B. E., Bernestein L., Ross R. K., (2000). Hormones and the etiology of cancer. IN: Cancer Medicine, 5th ed., Canada.

53. Hiragun A., (1985). Cell and tissue culture models of adipocyte development. In New Perspective in Adipose Tissue: Structure, Function and Development. Butterworth, London. 333-352.

54. Hiroi H, Inoue S, Watanabe T, et al. (1999). Differential immunolocalization of estrogen receptor alpha and beta in rat ovary and uterus. J Mol Endocrinol; 22: 37-44.

55. Hunter DJ, Spiegelman D, Adami HO, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Howe GR, Kushi LH, Marshall JR, Miller AB, Speizer FE, Willett W, Wolk A, Yaun SS. (1997). Non-dietary factors as risk factors for breast cancer, and as effect modifiers of the association of fat intake and risk of breast cancer. Cancer Causes Control.; 8: 49-56.

56. Jardé T, Perrier S, Vasson MP, Caldefie-Chézet F. (2011). Molecular mechanisms of leptin and adiponectin in breast cancer. Eur J Cancer 47:33-43.

57. Kadowaki T, Yamauchi T. (2005). Adiponectin and adiponectin receptors. Endocr Rev 26:439- 51.

58. Kang JH, Lee YY, Yu BY, Yang BS, Cho KH, Yoon DK, Roh YK. (2005). Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. Arch Pharm Res 28:1263-9.

59. Kang JH, Lee YY, Yu BY, Yang BS, Cho KH, Yoon DK, Roh YK. (2005). Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. Arch Pharm Res 28:1263-9.

60. Kelesidis I, Kelesidis T, Mantzoros CS. (2006). Adiponectin and cancer: a systematic review. Br J Cancer 94:1221-5.

61. Kershaw EE, Flier JS. (2004). Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89:2548- 56.

62. Key TJ, Verkasalo PK, Banks E. (2001). Epidemiology of breast cancer. Lancet Oncol. 2: 133–140.

63. Körner A, Pazaitou-Panayiotou K, Kelesidis T, Kelesidis I, Williams CJ, Kaprara A, Bullen J, Neuwirth A, Tseleni S, Mitsiades N, et al. (2007). Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. J Clin Endocrinol Metab 92:1041-8.

64. Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, et al. (2002). Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem 277:25863-6

65. Lahmann, P.H., Hoffmann, K., Allen, N., van Gils, C. H., Khaw, K. T., Tehard, B., Berrino, F., Tjonneland, A., Bigaard, J., Olsen, A., Overvad, K., Clavel-Chapelon, F., Nagel, G., Boeing, H., Trichopoulos, D., Economou, G., Bellos, G., Palli, D., Tumino, R., Panico, S., Sacerdote, C., Krogh, V., Peeters, P. H., Bueno-de-Mesquita, H. B., Lund, E., Ardanaz, E., Amiano, P., Pera, G., Quiros, J. R., Martinez, C., Tormo, M. J., Wirfalt, E., Berglund, G., Hallmans, G., Key, T. J., Reeves, G., Bingham, S., Norat, T., Biessy, C., Kaaks, R. and Riboli, E. (2004). Body size and breast cancer risk: Findings from the european prospective investigation into cancer and nutrition (EPIC). Int J Cancer, 111(5), 762-771.

66. Lanzino M, Morelli C, Garofalo C, Panno ML, Mauro L, Andò S, Sisci D. (2008). Interaction between estrogen receptor alpha and insulin/IGF signaling in breast cancer. Curr Cancer Drug Target 8:597-610.

67. Lee M-H, Klein RL, El-Shewy HM, Luttrell DK, Luttrell LM. (2008). The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/ Ras-dependent pathway and stimulate cell growth. Biochemistry 47:11682-92.

68. Lehrer S, Rabin J, Kalir T, Schachter B. (1993) Estrogen receptor variant and hypertension in women. Hypertension; 21: 439-441.

69. Lehrer S, Rabin J, Stone J, Berkowitz GS. (1994). Association of an estrogen receptor variant with increased height in women. Horm Metab Res; 26: 486-488.

70. Liu Q, Wuu J, Lambe M, Hsieh SF, Ekbom A, Hsieh CC. (2002). Transient increase in breast cancer risk after giving birth: postpartum period with the highest risk (Sweden) Cancer Causes Control.; 13: 299-305.

71. Lorincz, A.M. and Sukumar, S. (2006). Molecular links between obesity and breast cancer. Endocr. Relat. Cancer, 13(2), 279-292

72. Lottering M.L., Haay M., Seegers J.C. (1992). Effect of 17-β-E2 metabolities on the events of cell cycle on MCF-7 cells. Cancer Res. 52:5926-5932.

73. Luttrell LM. (2003).'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors. J Mol Endocrinol 30(2):117-26.

74. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, et al. (2002). Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat Med 8:731-7.

75. Mantzoros C, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, Papadiamantis Y, Markopoulos C, Spanos E, Chrousos G, et al. (2004). Adiponectin and breast cancer risk. J Clin Endocrinol Metab 89:1102-7.

76. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, et al. (2006). APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol 8:516-23.

77. Matsubara M, Maruoka S, Katayose S. (2002). Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women. Eur J Endocrinol 147:173-80.

78. Mauro L, Catalano S, Bossi G, Pellegrino M, Barone I, Morales S, Giordano C, Bartella V, Casaburi I, Andò S. (2007). Evidences that leptin up-regulates E-cadherin expression in breast cancer: effects on tumor growth and progression. Cancer Res 67:3412-21.

79. Mauro L, Pellegrino M, Lappano R, Vivacqua A, Giordano F, Palma MG, Andò S, Maggiolini M. (2010). E-cadherin mediates the aggregation of breast cancer cells induced by tamoxifen and epidermal growth factor. Breast Cancer Res Treat 121(1):79-89.

80. Mauro L, Surmacz E. (2004). IGF-I receptor, cell-cell adhesion, tumour development and progression. J Mol Histol 35:247-53.

81. Mauro L., Pellegrino M., De Amicis F., Ricchio E., Giordano F., Rizza P., Catalano S., Bonofiglio D., Sisci D., Panno M.L., Andò, S. (2014). Evidences that estrogen receptor α interferes with adiponectin effects on breast cancer cell growth. Cell Cycle 13,553-564.

82. Minokoshi Y. & Kim Y. B., (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinasi. Nature. 415, 339-343.

83. Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, Noguchi S. (2003). Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 9:5699-704.

84. Mizunuma H, Hosoi T, Okano H, Soda M, Tokizawa T, Kagami I,Miyamoto S, I buki Y, Inoue S, Shiraki M, Ouchi Y. (1997). Estrogen receptor gene polymorphism and bone mineral density at the lumbar spine of pre- and postmenopausal women. Bone; 21:379-383.

85. Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A, Watson PH. (1997). Estrogen receptor variants and mutations. J Steroid Biochem Mol Biol; 62:363-72.

86. Murphy LC, Weitsman GE, Skliris GP, Teh EM, Li L, Peng B, Davie JR, Ung K, Niu YL, Troup S, et al.(2006). Potential role of estrogen receptor alpha (ERalpha) phosphorylated at Serine118 in human breast cancer in vivo. J Steroid Biochem Mol Biol 102:139- 46.

87. Neville M.C., McFadden T.B., Forsyth I., (2002). Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia. 7:49-66.

88. Nussey S.S. & Whitehead, S.A. (2001). Endocrinology: An Integrated Approach, chapter 5: The gonad, London: Taylor & Francis.

89. Obeid S. & Hebbard L. (2012) Role of adiponectin and its receptors in cancer. Cancer Biol. Med. 9, 213-220.

90. Osborne C.K. & Arteaga C.L. (1990). Autocrine and paracrine growth regulation of breast cancer. Clinical implications. Breast Cancer Res Treat. 15: 3-11.

91. Panno ML, Giordano F, Rizza P, Pellegrino M, Zito D, Giordano C, Mauro L, Catalano S, Aquila S, Sisci D, et al. (2012). Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination. Breast Cancer Res Treat 136:443-55.

92. Patani N., Martin L.A., Dowsett M. (2013) Biomarkers for the clinical management of breast cancer: international perspective. Int. J. Cancer. 133, 1-13.

93. Pearce ST, V. Craig Jordan. (2004). The biological role of estrogen receptors α and β in cancer. Oncology Hematology; 50: 3-22.

94. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22:153-83.

95. Pearson O.H., Manni A., Arafah, B.M. (1982). Antiestrogen treatment of breast cancer: An overview. Cancer Res., 42(8 Suppl), 3424s-3429s.

96. Pfeiler GH, Buechler C, Neumeier M, Schäffler A, Schmitz G, Ortmann O, Treeck O. (2008). Adiponectin effects on human breast cancer cells are dependent on 17-beta estradiol. Oncol Rep 19(3):787-93.

97. Pike MC, Spicer DV, Dahmoush L, Press MF. Rev. (1993). Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol; 15: 17-35.

98. Poola I, Koduri S, Chatra S, Clarke R. (2000). Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. J Steroid Biochem Mol Biol; 72: 249-58.

99. Rose D.P., Komninou D., Stephenson G.D. (2004). Obesity, adipocytokines, and insulin resistance in breast cancer. Obesity Reviews 5 153-165.

100. Rose, D.P. & Vona-Davis, L. (2009) Influence of obesity on breast cancer receptor status and prognosis. Expert Rev. Anticancer Ther., 9(8), 1091-1101.

101. Rosen, E.D. & B.M. Spiegelman,(2006). Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444(7121):847-53.

102. Rovito D, Giordano C, Vizza D, Plastina P, Barone I, Casaburi I, Lanzino M, De Amicis F, Sisci D, Mauro L, et al.(2013). Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. J Cell Physiol 228:1314-22.

103. Roy P.G., & Thompson A.M. (2006). Cyclin D1 and breast cancer. Breast 15, 718-727.

104. Russo J, Wilgus G, Russo IH. (1979). Susceptibility of the mammary gland to carcinogenesis: I. Differentiation of the mammary gland as determinant of tumor incidence and type of lesion. Am J Pathol; 96:721-736.

105. Sar M, Welsch F. (1999). Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology; 140: 963-71.

106. Schachter BS, Lehrer. (1994). Risk of miscarriage and a common variant of the estrogen receptor gene. Am J Epidemiol; 140:1144-1145.

107. Sflomos G. & Brisken C. (2013). A new Achilles Heel in breast cancer? Oncotarget 4:1126-7

108. Short TH, Rothschild MF, Southwood OI, McLaren DG, De Vries A, Van Der Steen H, Eckardt GR, Tuggle CK, Helm J, Vaske DA, Mileham AJ, Plastow G. (1997). Effect of the estrogen receptor locus on reproduction and production traits in four commercial pig lines. J Anim Sci 75: 3138-3142.

109. Simoncini T. & Genazzani A.R. (2003). Non-genomic actions of sex steroid hormones. Eur J Endocrinol 148:281-92.

110. Song RX, Chen Y, Zhang Z, Bao Y, Yue W, Wang JP, Fan P, Santen RJ. (2010). Estrogen utilization of IGF- 1-R and EGF-R to signal in breast cancer cells. J Steroid Biochem Mol Biol 118:219-30.

111. Stephenson G.D. & Rose, D.P. (2003). Breast cancer and obesity: An update. Nutr. Cancer, 45(1), 1-16.

112. Su A.I., Wiltshire T., Batalov S., Lapp H., Ching K.A., Block D., Zhang J., Soden R., Hayakawa M., Kreiman G., Cooke M.P., Walker J.R., Hogenesch J.B., (2004). A gene atlas of the mouse and human protein- encoding transcriptomes. Proc Natl Acad Sci USA. 101:6062-7.

113. Szanto I. & Kahn C.R. (1999). Selective interaction between leptin and insulin signaling pathways in a hepatic cell line. Pro. Natl. Acad. Sci. USA. 97:2355-2360.

114. Tilg H, Moschen AR. (2006). Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 6:772-83.

115. Toniolo, P.G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K. L., Shore, R. E., Strax, P. and Pasternack, B. S. (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J. Natl. Cancer Inst., 87(3), 190-197.

116. Trayhurn P, Wood IS. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr. 92(3):347-355.

117. Treeck O, Lattrich C, Juhasz-Boess I, Buchholz S, Pfeiler G, Ortmann O. (2008). Adiponectin differentially affects gene expression in human mammary epithelial and breast cancer cells. Br J Cancer 99:1246- 50.

118. Trujillo ME, Scherer PE. (2005) Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 257:167-75.

119. van Marken Lichtenbelt, W.D., et al. (2009). Cold-activated brown adipose tissue in healthy men. N Engl J Med,360(15):1500-8.

120. Virtanen K.A., et al. (2009). Functional brown adipose tissue in healthy adults. N Engl J Med, 360(15): 1518-25.

121. Wahli W. & Martinez E. (1991). Superfamily of steroid nuclear receptors. Positive and negative regulators of gene expression. FASEB J. 5: 2243-9.

122. Walter P., Green S., Greene G., et al. (1985). Cloning of the human estrogen receptor cdna. Proc Natl Acad Sci USA; 82: 7889-7893.

123. Watanabe T., Inoue S., Sumito O. (1997). Agonistic effect of tamoxifen independent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptor α and β. Biochem Biophys Res Commun. 236:140-145.

124. Weihua Z., Warner M., Gustafsson J.A. (2002). Estrogen receptor beta in the prostate. Mol Cell Endocrinol; 193: 1-5.

125. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 86:1930-5.

126. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, et al. (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423:762-9.

127. Zhang Y., Proenca R., Maffei M., Barone M., Leopold L., Friedman J.M., (1994). Positional cloning of the mouse obese gene and its human homologue. Nature. 372,425-432.

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

Cancer Research

GPER Mediates Activation of HIF1α/VEGF Signaling by Estrogens

Ernestina Marianna De Francesco[1], Michele Pellegrino[1], Maria Francesca Santolla[1], Rosamaria Lappano[1], Emilia Ricchio[1], Sergio Abonante[2], and Marcello Maggiolini[1]

Abstract

Biological responses to estrogens in normal and malignant tissues are mainly mediated by the estrogen receptors ERα and ERβ, which function as ligand-activated transcription factors. In addition, the G protein–coupled receptor GPR30 (GPER) mediates estrogenic signaling in breast cancer cells and cancer-associated fibroblasts (CAF) that contribute to cancer progression. In this study, we evaluated the role elicited by GPER in the estrogen-regulated expression and function of vascular endothelial growth factor (VEGF) in ER-negative breast cancer cells and CAF. We demonstrated that 17β-estradiol (E2) and the GPER-selective ligand G-1 triggered a GPER/EGFR/ERK/c-fos signaling pathway that leads to increased VEGF via upregulation of HIF1α. In further extending the mechanisms involved in E2-supported angiogenesis, we also showed that conditioned medium from CAF treated with E2 and G-1 promoted human endothelial tube formation in a GPER-dependent manner. *In vivo*, ligand-activated GPER was sufficient to enhance tumor growth and the expression of HIF1α, VEGF, and the endothelial marker CD34 in a mouse xenograft model of breast cancer. Our findings offer important new insights into the ability of estrogenic GPER signaling to trigger HIF1α-dependent VEGF expression that supports angiogenesis and progression in breast cancer. *Cancer Res; 74(15); 4053–64. ©2014 AACR.*

Introduction

Breast cancer is the most frequently diagnosed malignancy in women in the United States and the leading cause of cancer-related death in women worldwide (1). Although the molecular mechanisms involved in breast tumor development remain to be fully understood, it has been established that E2 triggers stimulatory effects by binding to the estrogen receptor (ER)α and ERβ that regulate the expression of genes involved in cell-cycle progression, cell migration, and survival (2, 3). In addition, the G protein–coupled receptor (GPR)30/GPER has been shown to mediate estrogenic signaling in different normal and malignant cell contexts, including breast cancer (3–7). In this regard, the identification of selective GPER agonists or antagonists (8–12) has allowed the evaluation of certain biological responses elicited through GPER. Actually, GPER activates a network of transduction pathways involving the epidermal growth factor receptor (EGFR), the intracellular cyclic AMP (cAMP), the mitogen-activated protein kinases (MAPK) cascade, and calcium mobilization (13–15). Moreover, the potential of GPER to mediate growth effects in diverse types of tumors has been evidenced (6, 7) together with its involvement in the estrogen responsiveness of ER-negative breast cancer cells (16). In accordance with these findings, the expression of GPER was associated with aggressive features and lower survival rates in patients with endometrial and ovarian cancer (17, 18), whereas GPER was negatively correlated with relapse-free survival and positively associated with the resistance to tamoxifen treatment in breast tumors (19). Therefore, GPER may be considered as a prognostic marker and a further player involved to some extent in the failure of endocrine therapy in estrogen-sensitive malignancies (20). In addition, the GPER/EGFR signaling mediates the expression of cell-cycle regulatory genes in cancer-associated fibroblasts (CAF) derived from patients with breast tumor, suggesting that the action of GPER may involve a functional interaction between these main components of the tumor microenvironment and cancer cells (21).

Tumor angiogenesis is a complex process initiated by paracrine signals occurring through tumor cells and the surrounding stroma (22). As the vascular endothelial growth factor-A (VEGF-A, also referred to as VEGF) mainly drives cancer progression upon hypoxic conditions (23), VEGF inhibitors are currently used in different chemotherapeutic strategies (24). Hence, great efforts are still addressed toward a deeper understanding of the transduction mechanisms involved in the expression and function of VEGF. In this regard, it should be mentioned that ERα mediates the upregulation of VEGF by 17β-estradiol (E2) in estrogen-sensitive tumors (25, 26). In addition, E2 induced through ERα the expression of hypoxia inducible factor-1α (HIF1α), which is an acknowledged VEGF

[1]Department of Pharmacy, Health and Nutrition Sciences, University of Calabria, Rende (CS), Italy. [2]Regional Hospital, Cosenza, Italy.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Marcello Maggiolini, Department of Pharmacy, Health and Nutrition Sciences, University of Calabria, 87036 Rende (CS), Italy. Phone: 39-0984493076, Fax: 39-0984493458; E-mail: marcellomaggiolini@yahoo.it

doi: 10.1158/0008-5472.CAN-13-3590

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

regulator (27, 28). Nevertheless, it remains to be elucidated the potential of E2 in stimulating VEGF expression and angiogenesis in cell contexts lacking ER expression.

On the basis of the aforementioned data and our previous investigation showing that HIF1α/GPER signaling mediates the induction of VEGF by hypoxia in different model systems (29), in this study we have assessed the ability of ligand-activated GPER to regulate VEGF in ER-negative breast cancer cells as well as in CAFs and breast tumor xenografts. Our results provide novel insights into the ability of estrogens to induce VEGF expression and angiogenesis through GPER, hence extending the mechanisms through which estrogens trigger breast cancer progression.

Materials and Methods

Reagents

17β-Estradiol (E2), β-estradiol 6-(O-carboxy-methyl)oxime: BSA (E2-BSA), and 4-hydroxytamoxifen (OHT) were purchased from Sigma-Aldrich Srl. 1-[4-(-6-Bromobenzol[1,3]diodo-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c-] quinolin8yl] ethanone (G-1) and (3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G-15) were obtained from Tocris Bioscience. Tyrphostin AG1478 (AG) was purchased from Biomol Research Laboratories. PD98059 (PD) was obtained from Calbiochem. Human VEGF was purchased from Peprotech. All compounds were dissolved in dimethyl sulfoxide (DMSO) except E2 and OHT, which were solubilized in ethanol, and VEGF and E2-BSA, which were solubilized, respectively, in water and phosphate-buffered saline (PBS). Before use, E2-BSA stock solution was mixed with dextran (0.05 mg/mL) and charcoal (50 mg/mL) for 30 minutes, centrifuged at 3,000 × *g* for 10 minutes and passed through a 0.22-mm filter to remove the potential contamination of free E2.

Cell cultures

CAFs obtained from breast malignancies were characterized and maintained as we previously described (29). The SkBr3 breast cancer cells were obtained by ATCC, used less than 6 months after resuscitation, and maintained in RPMI-1640 without phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin/streptomycin (Life Technologies). Human umbilical vein endothelial cells (HUVEC), kindly provided by Dr. A. Caruso (University of Brescia, Brescia, Italy) and routinely tested and authenticated according to the ATCC suggestions, were seeded on collagen-coated flasks (Sigma-Aldrich Srl) and cultured in endothelial growth medium (EGM; Lonza), supplemented with 5% FBS (Lonza). Breast MCF-7 and prostate LNCaP cancer cells were obtained by ATCC, used less than 6 months after resuscitation, and maintained respectively in DMEM and RPMI-1640, both supplemented with 10% FBS and 100 μg/mL penicillin/streptomycin (Life Technologies). Cells were switched to medium without serum 24 hours before experiments.

Gene reporter assays

The 2.6-kb VEGF promoter-luciferase construct containing full-length VEGF promoter sequence (22,361 to +298 bp relative to the transcription start site) used in luciferase assays was a kind gift from Dr. P. Soumitro (Harvard Medical School, Boston, MA). The hypoxia responsive element (HRE)-mutated VEGF promoter (pVEGF-mut) reporter gene was kindly provided by Dr. J. Cheng (Shanghai Jiao Tong University School of Medicine, Shanghai, China). CAFs and SkBr3 cells (1×10^5) were plated into 24-well dishes with 500 μL/well culture medium containing 10% FBS and transfected for 24 hours with control shRNA, shHIF1α, and shGPER. A mixture containing 0.5 μg of reporter plasmid and 10 ng of pRL-TK was then transfected by using X-treme GENE 9 DNA Transfection Reagent, as recommended by the manufacturer (Roche Diagnostics). After 8 hours, cells were treated for 18 hours with E2 and G-1 in serum-free medium. Luciferase activity was measured with Dual Luciferase Kit (Promega) and normalized to the internal transfection control provided by *Renilla* luciferase. The normalized relative light unit values obtained from cells treated with vehicle were set as 1-fold induction, upon which the activity induced by treatments was calculated.

Gene expression studies

Total RNA was extracted and cDNA was synthesized by reverse transcription as previously described (29). The expression of selected genes was quantified by real-time PCR using Step One sequence detection system (Applied Biosystems Inc.). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc.). For *ERα, ERβ, c-fos, HIF1α, VEGF*, and the ribosomal protein *18S*, the primers were: ERα Fwd: 5′-AGAGGGCATGGTGGAGATCTT-3′ and Rev: 5′-CAAACTCCTCTCCCTGCAGATT-3′; ERβ Fwd: 5′-GACCACAAGCCCAAATGTGTT-3′ and Rev: 5′-ACTGGCGATGGACCACTAAA-3′; c-fos: Fwd: 5′-GAGCCCTTTGATGACTTCCT-3′ and Rev: 5′-GAGCGGGCTGTCTCAGA-3′; HIF1α Fwd: 5′-TGCATCTCCATCTTCTACCCAAGT-3′ and Rev: 5′-CCGACTGTGAGTGCCACTGT-3′; VEGF: Fwd: 5′-TGCAGATTATGCGGATCAAACC-3′ and Rev: 5′-TGCATTCACATTTGTTGTGCTGTAG-3′; 18S Fwd: 5′-GGCGTCCCCCAACTTCTTA-3′ and Rev: 5′-GGGCATCACAGACCTGTTATT-3′. Assays were performed in triplicate and the RNA expression values were normalized using 18S expression and then calculated as fold induction.

Western blot analysis

SkBr3 cells, CAFs, and tumor homogenates obtained from nude mice were processed according to a previous described protocol (29). Protein lysates were electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel, electroblotted onto a nitrocellulose membrane and probed with primary antibodies against HIF1α (R&D Systems, Inc.), ERβ (Serotec), ERα (F-10), GPER (N-15), CD34 (ICO115), phosphorylated ERK1/2 (E-4), ERK2 (C-14), phosphorylated EGFR (Tyr 1173), EGFR (1005), and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA). Proteins were detected by horseradish peroxidase–linked secondary antibodies (Santa Cruz Biotechnology, DBA) and revealed using the ECL System (GE Healthcare).

Gene silencing experiments and plasmids

Cells were plated onto 10-cm dishes and transfected by X-treme GENE 9 DNA Transfection Reagent (Roche Molecular

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

Biochemicals) for 24 hours before treatments with a control vector, a specific shRNA sequence for each target gene, the plasmid DN/c-fos encoding a c-fos mutant that heterodimerizes with c-fos dimerization partners but not allowing DNA binding (kindly obtained from Dr. C. Vinson, NIH, Bethesda, MD; ref. 30). The HIF1α shRNA and the respective control plasmid were purchased from SABioscience Corporation. The silencing of GPER expression was obtained by a construct previously described (31).

Immunofluorescence microscopy

SkBr3 cells and CAFs were grown on coverslips, then serum deprived and transfected for 24 hours with shHIF1α or shGPER and the respective control plasmids. Thereafter, cells were treated for 18 hours with E2 and G-1, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed 3 times with PBS, and incubated overnight with a mouse primary antibody against VEGF (C-1; Santa Cruz Biotechnology, DBA). After incubation, the slides were extensively washed with PBS and incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), (1:1,000; Sigma-Aldrich) and donkey anti-mouse IgG-FITC (1:300; purchased from Alexa Fluor; Life Technologies). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS) were used for the microscopy evaluation.

Conditioned medium

CAFs were cultured in regular growth medium, then cells were washed twice with PBS and transfected for 24 hours in serum-free RPMI-1640 with shGPER or control shRNA using X-treme GENE 9 DNA Transfection Reagent as recommended by the manufacturer (Roche Molecular Biochemicals). Cells were treated for 18 hours with E2 and G-1, the culture medium was then replaced for additional 18 hours with medium without serum and treatments. Thereafter, the supernatants were collected, centrifuged at 3,500 rpm for 5 minutes to remove cell debris, and used as conditioned medium in HUVECs.

Evaluation of E2 production

SkBr3 and CAFs cultured in regular growth medium were rinsed twice with PBS and incubated with serum-free medium for 24 hours. Culture supernatants were collected and centrifuged at 3,500 rpm for 5 minutes to remove cell debris. Three hundred pg/mL of E2 were added to collected supernatants in order to obtain a positive control. E2 production was measured by ELISA (Enzo Life Sciences) in three independent experiments performed in triplicate.

Tube formation assay

The day before the experiment, HUVECs were cultured in serum-free medium (EBM; Lonza). Growth factor–reduced Matrigel (Cultrex; Trevigen Inc.) was thawed overnight at 4°C on ice, plated on the bottom of prechilled 96-well plates, and left at 37°C for 1 hour for gelification. Starved HUVECs were collected by enzymatic detachment (0.25% trypsin-EDTA solution; Life Technologies), counted, and resuspended in conditioned medium from CAFs. Then, 10,000 cells/well were seeded on Matrigel and incubated at 37°C. Cord formation was observed 2 hours after cell seeding. Tube formation was quantified by using the software NIH ImageJ.

Proliferation assay

For quantitative proliferation assay, SkBr3 cells (1×10^5) were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped FBS with the indicated treatments; medium was renewed every 2 days (with treatments) and cells were counted using the Countess Automated Cell Counter, as recommended by the manufacturer's protocol (Life Technologies).

In vivo studies

Female 45-day-old athymic nude mice (nu/nu Swiss; Harlan Laboratories) were maintained in a sterile environment. At day 0, exponentially growing SkBr3 cells (8.0×10^6 per mouse) were inoculated subcutaneously in 0.1 mL of Matrigel (Cultrex; Trevigen Inc.). When the tumors reached average ~0.15 cm^3 (i.e., in about 1 week), mice were randomized and divided into four groups, according to treatments administered by intramuscular injection for 40 days. The first group of mice ($n = 7$) was treated daily with 100 μL of vehicle (0.9% NaCl with 0.1% albumin and 0.1% Tween-20; Sigma-Aldrich), the second group of mice ($n = 7$) was treated daily with 100 μL G-1 (0.5 mg/kg/die), the third group of mice ($n = 7$) was treated daily with 100 μL G-15 (3.5 mg/kg/die), and the fourth group of mice ($n = 7$) was treated daily with 100 μL G-1 in combination with G-15 (at the concentrations described above). G-1 and G-15 were dissolved in DMSO at 1 mg/mL. For treatments, 6.2 μL of G-1 were added to 93.8 μL of vehicle and 44 μL G-15 were added to 56 μL of vehicle. The doses of G-15 were chosen to represent an approximately 1:7-fold μg excess with respect to G-1. SkBr3 xenograft tumor growth was evaluated by caliper measurements, along two orthogonal axes: length (L) and width (W). Tumor volumes (in cm^3) were estimated by the following formula: $TV = L \times (W^2)/2$. At day 40, the animals were sacrificed following the standard protocols and tumors were dissected from the neighboring connective tissue. Specimens of tumors were frozen in nitrogen and stored at −80°C, the remaining tumor tissues of each sample were fixed in 4% paraformaldehyde and embedded in paraffin for the histologic analyses. Animal care, death, and experiments were done in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and in accordance with the Italian law (DL 116, January 27, 1992). The project was approved by the local ethical committee.

Histologic analysis

Formalin-fixed, paraffin-embedded sections of tumor xenografts were cut at 5 μm and allowed to air dry. Deparaffinized, rehydrated sections were stained for 6 minutes with hematoxylin (Bio-Optica), washed in running tap water,

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

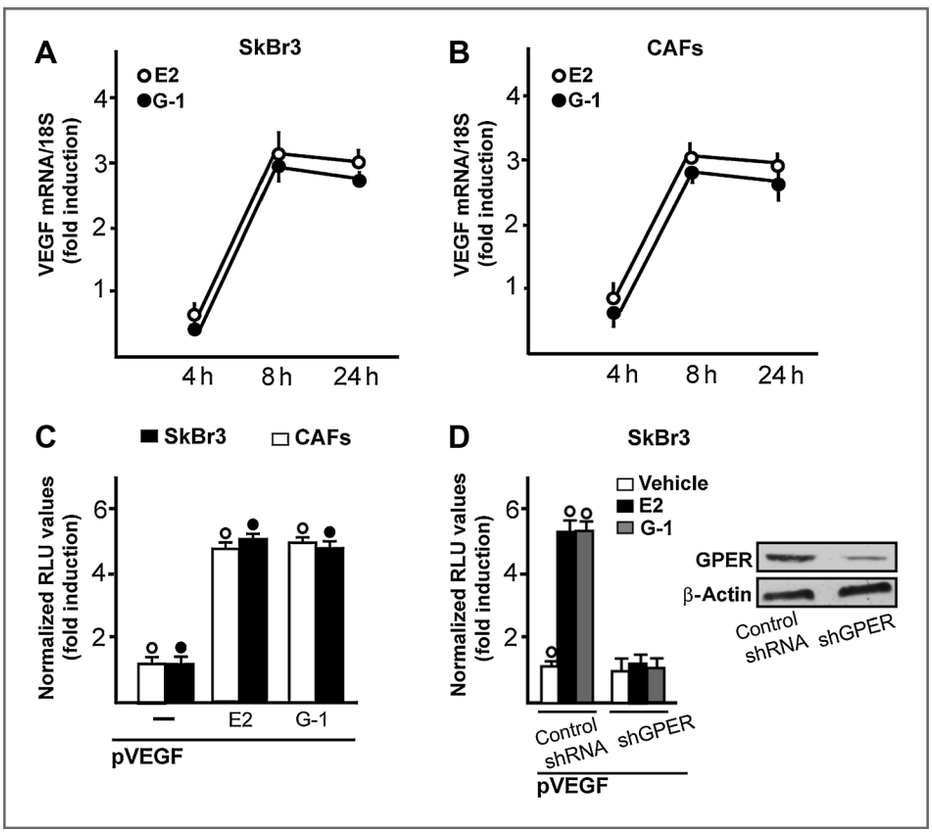

Figure 1. E2 and G-1 induce the expression of VEGF. In SkBr3 cells (A) and CAFs (B), 1 nmol/L E2 and 1 μmol/L G-1 upregulate the mRNA expression of *VEGF*, as evaluated by real-time PCR. Gene expression was normalized to 18S expression and results are shown as fold changes of mRNA expression compared with cells treated with vehicle. C, an expression vector encoding the VEGF promoter sequence (pVEGF) is transactivated in SkBr3 cells and CAFs treated for 18 hours with 1 nmol/L E2 and 1 μmol/L G-1. D, in SkBr3 cells, the transactivation of the pVEGF induced by a 18-hour treatment with 1 nmol/L E2 and 1 μmol/L G-1 is abrogated, silencing the expression of GPER. Side panel shows the efficacy of GPER silencing, as evaluated by immunoblotting. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction, upon which the activities induced by treatments were calculated. Each data point represents the mean ± SD of three independent experiments performed in triplicate. ○ and ●, $P < 0.05$ for cells receiving vehicle (–) versus treatments.

and counterstained with eosin Y (Bio-Optica). Sections were then dehydrated, cleared with xylene, and mounted with resinous mounting medium. Morphologic analyses were carried out on paraffin-embedded tumor sections, stained with hematoxylin and eosin (H&E), or immunolabeled with human cytocheratin 18 (Santa Cruz Biotechnology, DBA). Tumor sections were immunolabeled with CD34, which served as a vessel marker (32, 33).

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls' testing to determine differences in means. Statistical comparisons for *in vivo* studies were made using the Wilcoxon–Mann–Whitney test. $P < 0.05$ was considered statistically significant.

Results

E2 and G-1 induce VEGF expression through GPER

Considering the ability of estrogens to stimulate the expression of the proangiogenic mediator VEGF (25, 26), we aimed to evaluate whether GPER may be involved in this response to estrogens. To this end, we used as a model system the SkBr3 breast cancer cells and CAFs that do not express the classical ERs (Supplementary Fig. S1) and are not able to produce E2 following our experimental conditions (Supplementary Fig. S1). As determined by real-time PCR, the selective GPER agonist G-1 and E2 induced the mRNA expression of *VEGF* (Fig. 1A and B). Accordingly, G-1 and E2 transactivated a VEGF promoter construct (Fig. 1C) through GPER, as the luciferase activity was repressed knocking down the expression of GPER in SkBr3 cells (Fig. 1D) and CAFs (data not shown). The aforementioned findings were then confirmed by immunofluorescence experiments performed in both SkBr3 cells (Fig. 2) and CAFs (Supplementary Fig. S2), further corroborating the involvement of GPER in the upregulation of VEGF protein expression induced by estrogens in these cells.

HIF1α expression is regulated by estrogens through GPER along with the EGFR/ERK/c-FOS transduction pathway

On the basis of previous data showing that estrogens induce the expression of HIF1α, which has been largely involved in the transcriptional regulation of VEGF (27, 28), we asked whether this action of estrogens may occur through GPER. Notably, E2 and G-1 upregulated the mRNA expression (Fig. 3A and B) and the protein levels of HIF1α (Fig. 3C–F) in a GPER-dependent manner as the GPER silencing abrogated these responses (Fig. 3G and H and Supplementary Fig. S3). Next, the HIF1α protein increase by E2 and G-1 was abrogated in presence of the EGFR inhibitor AG1478 (AG), the MEK inhibitor PD, or using a plasmid encoding a c-fos mutant named dominant/negative c-fos (DN/c-fos) in both SkBr3 cells (Fig. 4A and B) and CAFs (Fig. 4C and D). Taken together, these data indicate that

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

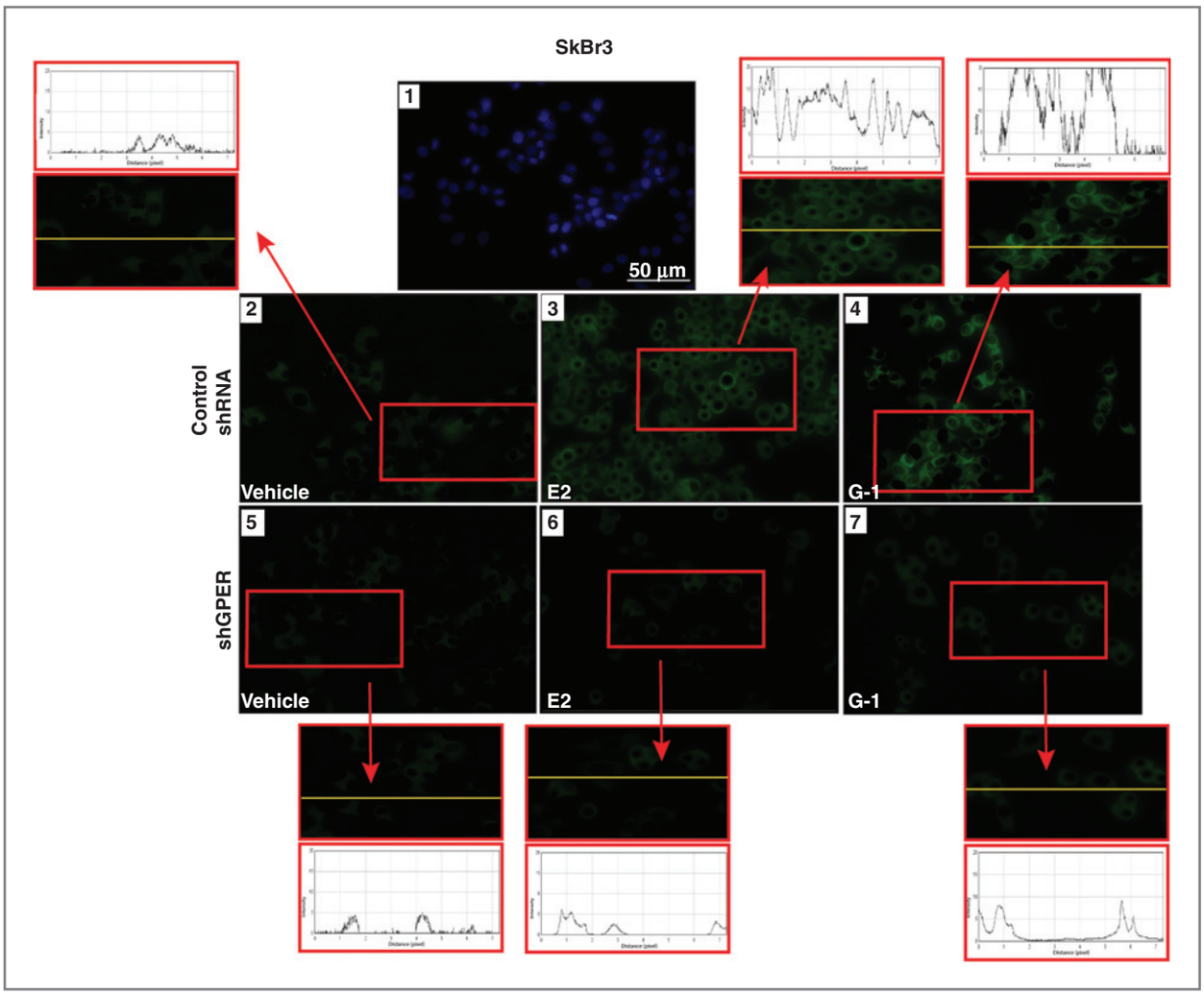

Figure 2. GPER mediates the upregulation of VEGF protein expression induced by E2 and G-1. Evaluation of VEGF protein expression by immunofluorescent microscopy in SkBr3 cells transfected for 24 hours with control shRNA (panels 2–4) or shGPER (panels 5–7). Cells were treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1, as indicated; the accumulation of VEGF is evidenced by the green signal. Nuclei were stained by DAPI (blue signal in panel 1). Using the program WCIF Image J for Windows for expressive analysis, the plot profiles obtained at the level of the yellow line of the corresponding inset are shown in each side panel. Note the high values indicating zones of intense labeling. Images shown are representative of 20 random fields of three independent experiments.

GPER along with the EGFR/ERK/c-fos transduction pathway mediate the HIF1α expression induced by E2 and G-1.

HIF1α is involved in the upregulation of VEGF induced by E2 and G-1

Considering that HIF1α exerts a crucial role in the regulation of VEGF in diverse pathophysiologic conditions, we aimed to evaluate whether the VEGF protein induction by E2 and G-1 is mediated by HIF1α. Performing immunofluorescence assays we ascertained that the upregulation of VEGF induced by these ligands is abrogated knocking down HIF1α expression in both SkBr3 cells (Fig. 5 and Supplementary Fig. S3) and CAFs (Supplementary Figs. S3 and S4). Accordingly, the transactivation of a VEGF promoter construct triggered by E2 and G-1 was prevented silencing HIF1α expression in SkBr3 cells (Supplementary Fig. S5) and CAFs (data not shown). Likewise, the luciferase activity induced by E2 and G-1 was prevented transfecting cells with a HRE-mutated VEGF promoter construct (Supplementary Fig. S5). It is worth noting that E2 and G-1 were also not able to stimulate VEGF expression transfecting both SkBr3 cells and CAFs with the DN/c-fos plasmid (data not shown).

Further supporting the aforementioned findings, in SkBr3 cells the membrane impermeable E2-BSA induced the EGFR and ERK1/2 phosphorylation (Supplementary Fig. S6) as well as the upregulation of c-fos, HIF1α, and VEGF mRNA expression (Supplementary Fig. S6). In addition, E2 and G-1 alone and in the presence of the ER antagonist OHT promoted the activation of EGFR and ERK1/2 (Supplementary Fig. S6) as well as the upregulation of *c-fos*, *HIF1α*, and *VEGF* mRNA

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

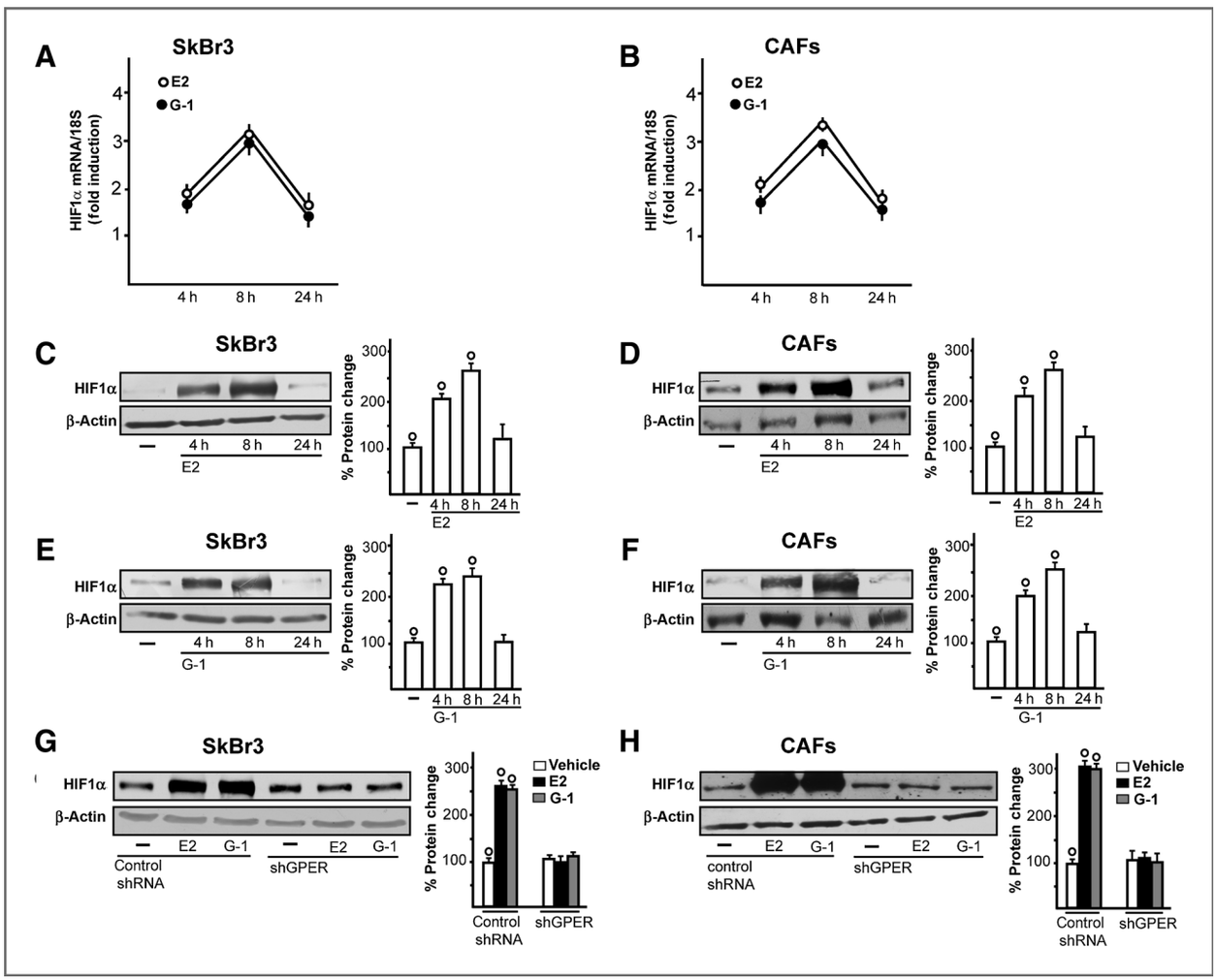

Figure 3. The HIF1α expression induced by E2 and G-1 occurs through GPER. In SkBr3 cells and CAFs, 1 nmol/L E2 and 1 μmol/L G-1 upregulate HIF1α expression at both mRNA (A and B) and protein levels (C–F), as indicated. SkBr3 (G) cells and CAFs (H) were transfected with control shRNA or shGPER for 24 hours and then treated for 12 hours with vehicle (–), 1 nmol/L E2, and 1 μmol/L G-1. Gene expression was evaluated by real-time PCR and normalized to 18S expression. Results are shown as fold changes of mRNA expression compared with cells treated with vehicle. Protein expression was evaluated by immunoblotting experiments and side panels show densitometric analysis of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. ○, $P < 0.05$ for cells receiving vehicle (–) versus treatments.

expression (Supplementary Fig. S6). It is worth noting that OHT stimulated the aforementioned responses (Supplementary Fig. S6) acting as a GPER agonist, according to previous studies (4, 7). Altogether, these data may suggest that GPER mediates the estrogen-induced expression of VEGF through the EGFR/ERK/c-fos transduction pathway and the involvement of HIF1α.

GPER is involved in VEGF-mediated tube formation induced by E2 and G-1

Having established that GPER mediates the estrogen-induced upregulation of VEGF, we analyzed the potential role of GPER in endothelial tubulogenesis. HUVECs that represent a useful model system for *in vitro* evaluation of neoangiogenesis (34) were cultured using conditioned medium from CAFs previously treated with E2 and G-1. Interestingly, a ramified network of tubules was generated in HUVECs grown in medium from CAFs treated with E2 and G-1 (Fig. 6A), whereas these ligands had no effects knocking down the expression of GPER in CAFs (Fig. 6B). Further supporting these data, the addition of VEGF to medium collected from GPER-silenced CAFs rescued the generation of tubule structures in HUVECs (Fig. 6C). These results, recapitulated in Fig. 6D–F, indicate that VEGF may be considered as a target of the estrogenic GPER signaling toward new blood vessels formation.

GPER mediates HIF1α and VEGF expression along with growth effects in breast cancer xenografts

Then, we turned to an *in vivo* model system. Female nude mice bearing into the intrascapular region SkBr3 cell tumor xenografts were treated with vehicle, G-1 alone, and in combination with the GPER antagonist G-15 (10). These

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

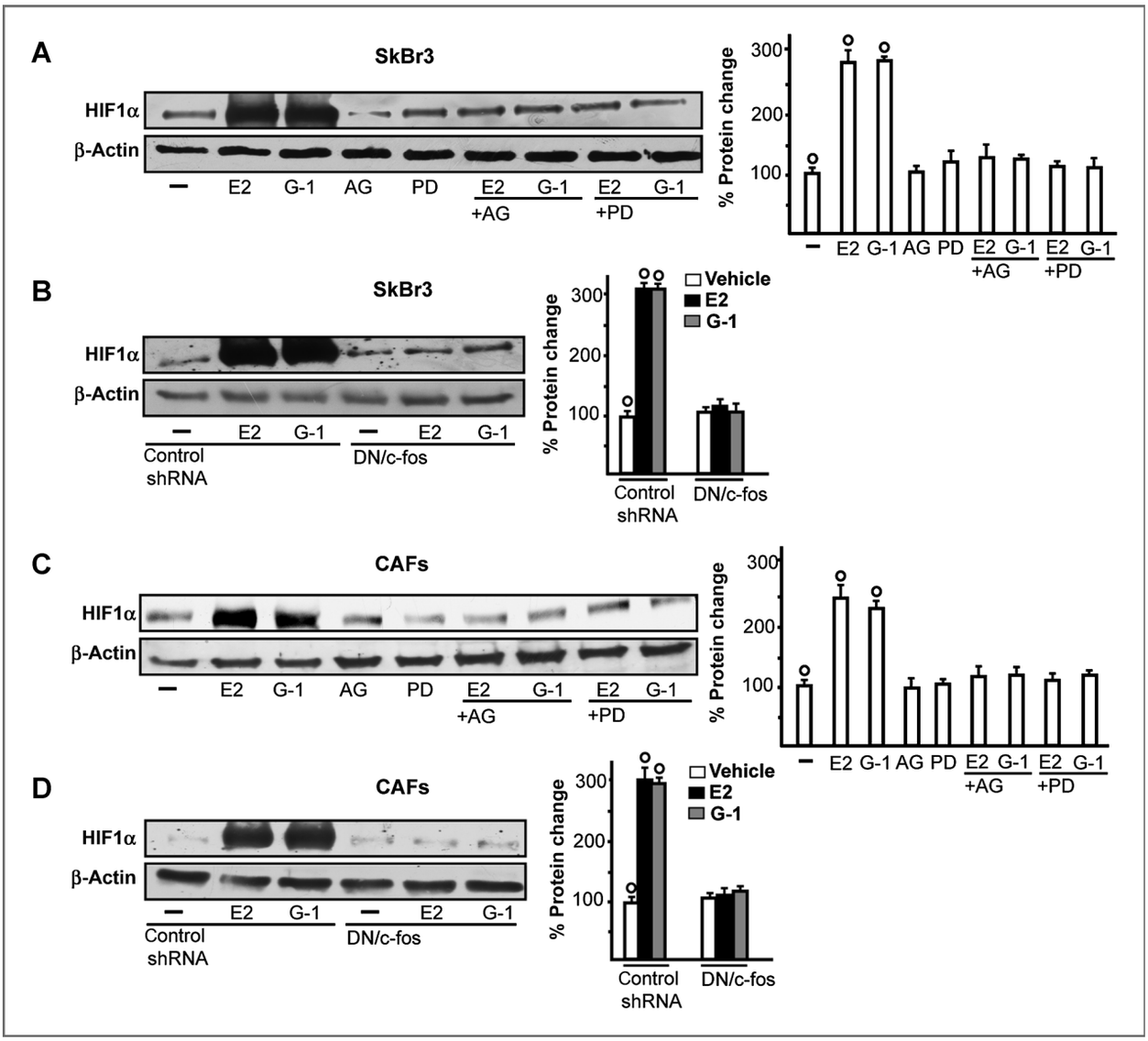

Figure 4. The EGFR/ERK/c-FOS transduction pathway mediates the HIF1α expression induced by E2 and G-1. Immunoblots showing HIF1α protein expression in SkBr3 cells (A) and CAFs (C) treated for 12 hours with vehicle (–), 1 nmol/L E2, and 1 μmol/L G-1 alone or in combination with 10 μmol/L EGFR inhibitor AG1478 (AG), 10 μmol/L MEK inhibitor PD98089 (PD). Evaluation of HIF1α protein expression in SkBr3 cells (B) and CAFs (D), which were transfected for 24 hours with control shRNA or a plasmid encoding for a dominant negative form of c-fos (DN/c-fos) and treated for 12 hours with vehicle (–), 1 nmol/L E2 and 1 μmol/L G-1. Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. ○, $P < 0.05$ for cells receiving vehicle (–) versus treatments.

administrations were well tolerated as no change in body weight or in food and water consumption was observed together with no evidence of reduced motor function. In addition, no significant difference in the mean weights or histologic features of the major organs (liver, lung, spleen, and kidney) was observed after sacrifice among vehicle and ligands-treated mice, thus indicating a lack of toxic effects. Histologic examination of SkBr3 xenografts revealed that tumors were primarily composed of tumor epithelial cells (Supplementary Fig. S7). After 40 days of treatment, the tumor growth induced by G-1 was prevented by G-15 (Fig. 7A). Representative tumor images are shown in Fig. 7B. As evidenced in Supplementary Fig. S6, E2 and G-1 stimulated the proliferation of SkBr3 cells also in growth assays performed *in vitro*. Next, we found increased HIF1α and VEGF protein levels in tumor homogenates obtained from G-1–stimulated mice with respect to vehicle-treated mice; however, these stimulatory effects were abrogated in the animal group receiving G-15 in addition to G-1 (Fig. 7C). The acknowledged marker of endothelial cell proliferation CD34 (32, 33) paralleled the increased expression of HIF1α and VEGF upon treatments (Fig. 7C and Supplementary Fig. S8), suggesting that ligand-activated GPER may stimulate tumor growth and angiogenesis.

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

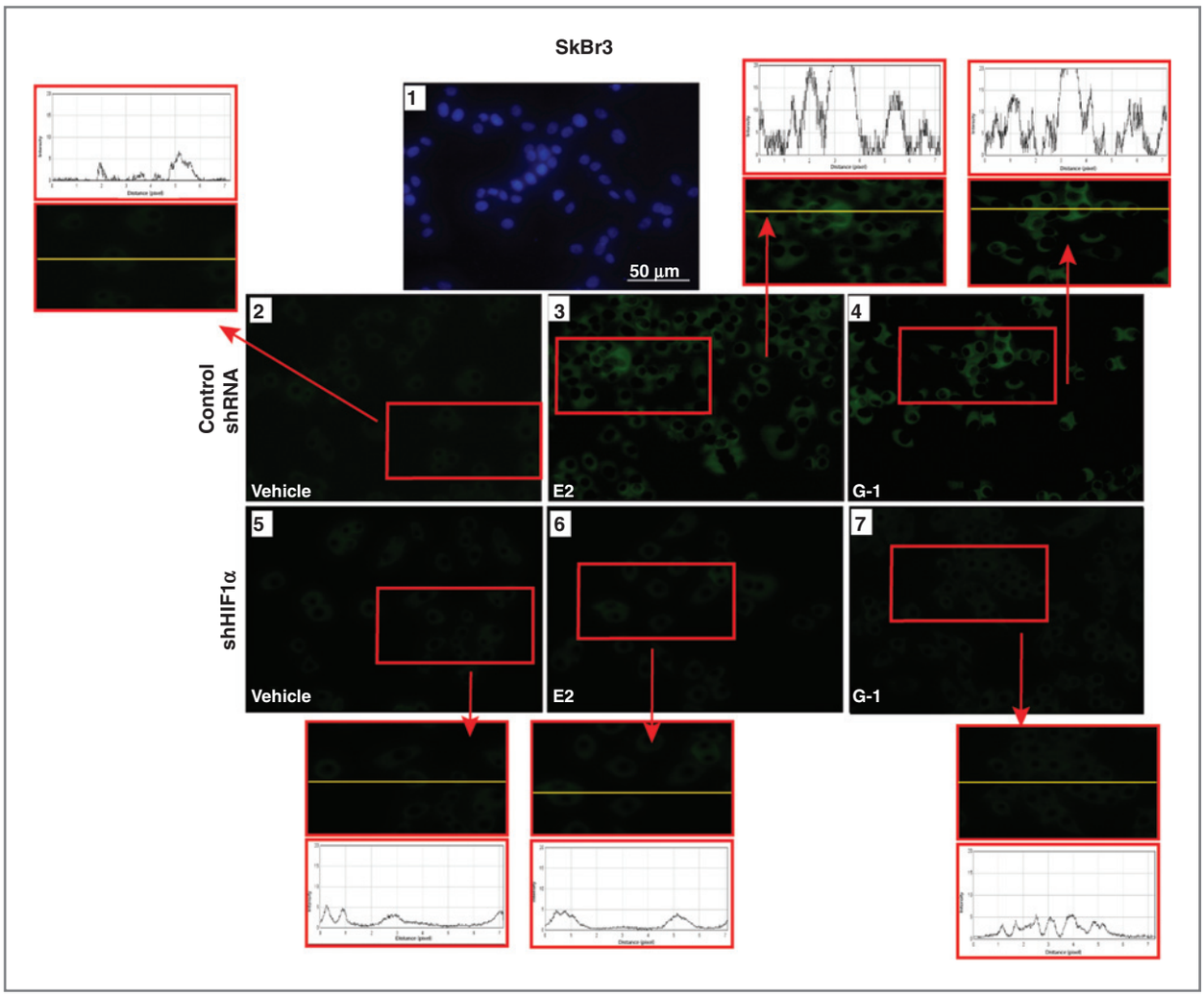

Figure 5. VEGF expression induced by E2 and G-1 occurs through HIF1α. Evaluation of VEGF expression by immunofluorescent microscopy in SkBr3 cells transfected for 24 hours with control shRNA (panels 2–4) or shHIF1α (panels 5–7). Cells were treated with vehicle, 1 nmol/L E2, and 1 μmol/L G-1 for 18 hours, as indicated. VEGF accumulation is evidenced by the green signal. Nuclei were stained by DAPI (blue signal in panel 1). Using the program WCIF Image J for Windows for expressive analysis, the plot profiles obtained at the level of the yellow line of the corresponding inset are shown in each side panel. Note the high values, indicating zones of intense labeling. Images shown are representative of 20 random fields of three independent experiments.

Discussion

In this study, we have demonstrated that GPER mediates the upregulation of VEGF expression induced by E2 and G-1 in breast cancer cells and CAFs. In particular, we have ascertained that GPER activation by both ligands engages the EGFR/ERK/c-fos transduction signaling toward the induction of HIF1α and VEGF transcription. As a biological counterpart, we have evidenced that GPER mediates endothelial tube formation in HUVECs cultured in medium from CAFs, which were previously treated with E2 and G-1. In addition, we have determined that G-1 induces growth effects in SkBr3 tumor xenografts and increases the expression of HIF1α, VEGF, and CD34 in tumor homogenates. Therefore, the present findings provide novel insights into the potential of GPER to mediate estrogen-dependent regulation and function of VEGF, which plays a main role in tumor angiogenesis and progression (23, 24).

Breast cancer is stimulated by estrogens that activating the classical ERs modulate cell proliferation, adhesion, migration, and invasion in estrogen-sensitive tumors (2, 3). In addition, estrogens promote the formation of new blood vessels within the tumor mass (35), hence suggesting that these steroids elicit a stimulatory role not only in cancer cells but also in components of the surrounding stroma in accordance with the results obtained in this study. As the complex process of angiogenesis is required for tumor progression, it represents a central biological target in cancer (36). VEGF is one of the most potent proangiogenic factor playing a paramount role in the formation of blood vessels in the development of different types of tumors, including breast cancer (23). Accordingly, VEGF is highly expressed in breast cancer specimens compared with normal breast tissue (37) and its suppression leads to the inhibition of breast tumor development (38). Diverse factors

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

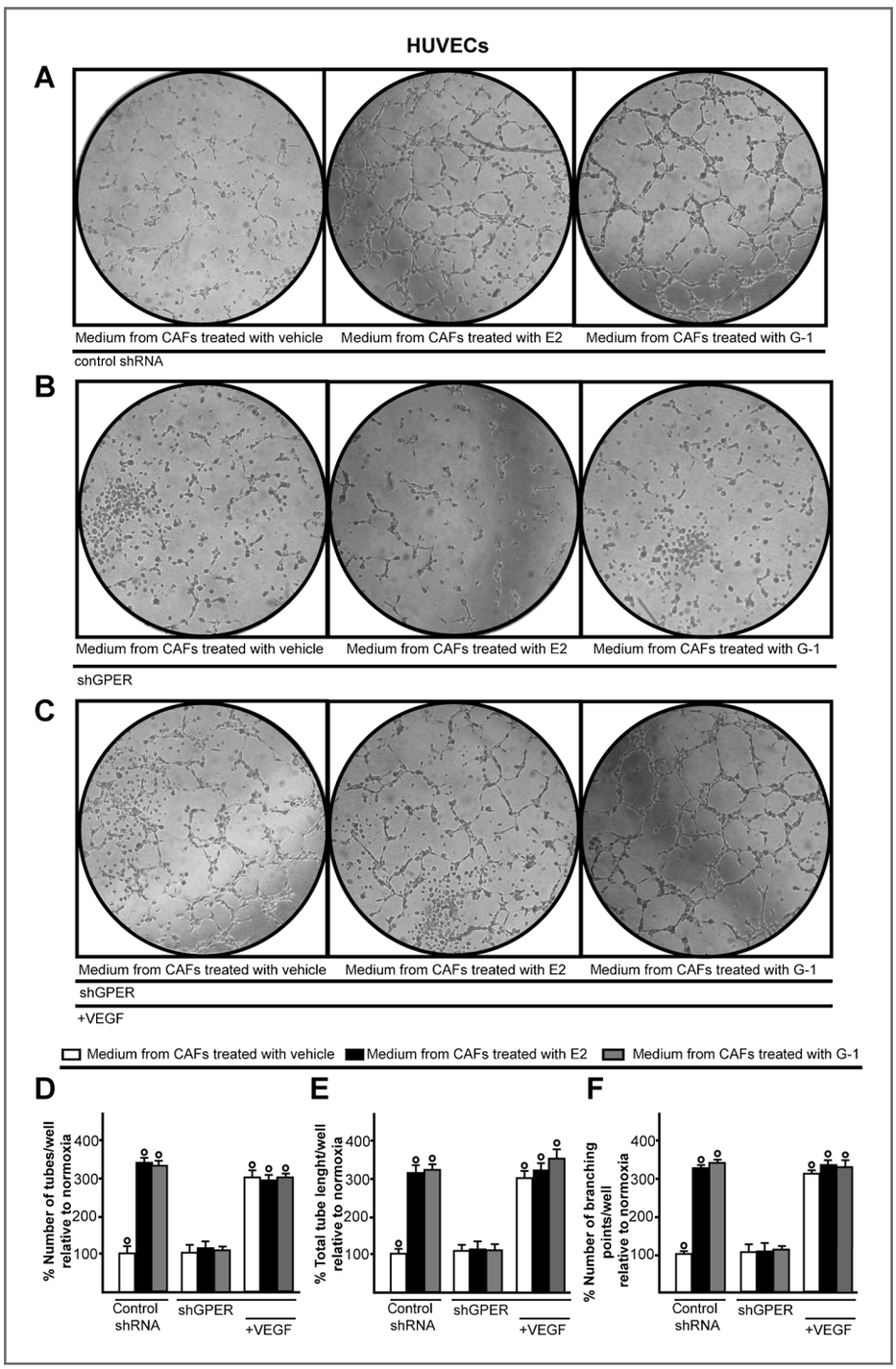

Figure 6. GPER mediates the endothelial tube formation triggered by E2 and G-1. Tube formation was evaluated in HUVECs cultured for 2 hours in medium collected from CAFs transfected for 24 hours with control shRNA (A) or shGPER (B) and then treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1, as indicated. C, tube formation is rescued using 10 ng/mL VEGF in HUVECs cultured in medium from CAFs, which were transfected for 24 hours with shGPER and then treated for 18 hours with vehicle, 1 nmol/L E2, and 1 μmol/L G-1. Quantification of the number of tubes (D), total tube length (E), and number of branching points (F) observed in HUVECs. Data are representative of three independent experiments performed in triplicate. ○, $P < 0.05$ for cells receiving medium of CAFs treated with vehicle versus medium of CAFs treated with ligands.

regulate VEGF expression like hypoxia, cytokines, growth factors, and hormones (23). In this regard, previous studies have demonstrated the ability of estrogens to upregulate VEGF levels activating ERα, which binds to the estrogen response elements (ERE) located within the VEGF promoter region (25, 26, 39). As these studies evaluated the mechanisms involved in the estrogen-regulated VEGF expression in ER-positive cells, in this study we evaluated the potential of estrogens to trigger the transcription of VEGF in ER-negative cells. In particular, using both *in vitro* and *in vivo* model systems we have provided novel evidence about the GPER-mediated stimulation of VEGF, although further investigations are needed to better clarify the role played by GPER in the modulation of VEGF in diverse pathophysiologic conditions.

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

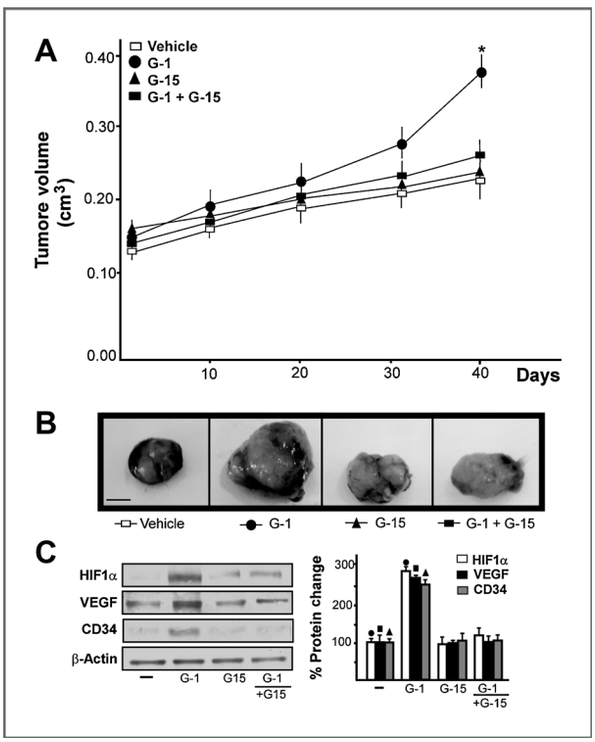

Figure 7. GPER is involved in SkBr3 cells xenograft growth. A, tumor volume from SkBr3 xenografts implanted in female athymic nude mice treated for 40 days with vehicle, G-1 (0.25 mg/kg/die), and G-15 (3.5 mg/kg/die) alone or in combination, as indicated. *, $P < 0.05$ for G-1–treated animals versus vehicle-treated animals. B, representative images of experimental tumors at day 40; scale bar, 0.3 cm. HIF1α, VEGF, and CD34 protein expression in tumor homogenates from SkBr3 xenografts treated as reported above (C). Side panels show densitometric analysis of the blots normalized to β-actin. ●, ■, ▲, $P < 0.05$ for G-1–treated animals versus vehicle-treated animals.

Interestingly, our data may recall previous findings showing that the ER antagonist and GPER agonist OHT (4, 7) is able to upregulate VEGF in different tumor types, including breast cancer (40, 41). In addition, it has been recently reported that low survival rates in patients with endometrial cancer are associated with both elevated GPER expression and VEGF levels (42), further providing a relationship between these 2 main players of tumor cells and the surrounding stroma.

HIF1α constitutes with HIF1β the active transcriptional complex HIF1 that regulates many genes involved in important biological functions in cancer cells like energy metabolism, survival, cell migration, and neovascularization (43). Cytokines, growth factors, and hormones beyond hypoxia were shown to upregulate the expression of HIF1α (44). For instance, in normoxic conditions activated EGFR increased HIF1α expression through the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and MAPK transduction pathways (45). Further extending these data, our present results indicate that the GPER/EGFR/ERK/c-fos transduction signaling is involved in the upregulation of HIF1α by estrogens, thus providing novel insights into the mechanisms mediating the HIF1α-dependent stimulation of VEGF. Moreover, the current results corroborate our previous studies showing that a cross-talk between HIF1α and GPER regulates the expression and function of VEGF upon hypoxic conditions (29).

The stromal contribution to the development of a wide variety of tumors has been extensively assessed using both *in vitro* and *in vivo* model systems (46, 47). For instance, it has been shown that malignant cells may recruit into the tumor mass diverse components of the microenvironment like CAFs, inflammatory and vascular cells that actively cooperate toward cancer progression (46, 47). In particular, increasing evidence has suggested that CAFs contribute to cancer aggressiveness through the production of secreted factors that target numerous stromal components and cancer cell types (46–48). In breast carcinomas, CAFs have been shown to elicit relevant biological activities including the stimulation of new blood vessels formation, which closely correlates with cancer growth, metastasis, and poor prognosis (29, 46–48). Our results further extend these findings as the medium collected from CAFs, which were stimulated by estrogens, induced tube formation in HUVECs. This response occurred through the GPER-mediated release of VEGF, suggesting that the paracrine signaling between CAFs and endothelial cells may trigger angiogenic processes toward tumor progression. Likewise, the growth effects observed in breast cancer xenografts upon ligand-activated GPER were paralleled in tumor homogenates by an increased expression of HIF1α, VEGF, and the vessel marker CD34. In line with these data, an enhanced tumor growth occurred *in vivo* using breast cancer cells engineered to express elevated levels of VEGF (49), on the contrary the inhibition of VEGF led to the growth arrest of breast carcinomas in nude mice (38). In addition, an increased VEGF expression upon E2 exposure was found in rat mammary cancer (50), further highlighting the main role played by VEGF in breast tumorigenesis.

The present data provide novel insights into the potential of estrogenic GPER signaling to trigger the HIF1-mediated increase of VEGF toward angiogenesis and cancer progression. Furthermore, the paracrine responses mediated by GPER extend the current knowledge on the critical interaction between cancer cells and the surrounding stroma, which plays a pivotal role in tumor development and metastasis. Altogether, these findings may be taken into account in setting novel therapeutic strategies targeting the stimulatory action of estrogens in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E.M. De Francesco, M. Maggiolini
Development of methodology: E.M. De Francesco, M.F. Santolla, R. Lappano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.M. De Francesco, M. Pellegrino, M.F. Santolla, E. Ricchio, S. Abonante
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.M. De Francesco, M. Pellegrino, E. Ricchio, M. Maggiolini
Writing, review, and or revision of the manuscript: E.M. De Francesco, M. Maggiolini
Study supervision: M. Maggiolini

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

Grant Support

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, project no. 12849/2012), AIRC project Calabria 2011 (http://www.airc.it/), and Fondazione Cassa di Risparmio di Calabria e Lucania. E.M. De Francesco was supported by Associazione Italiana per la Ricerca sul Cancro (biennial fellowship Isabella Adonio Curcio). E. Ricchio was supported by European Commission/FSE/Regione Calabria.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 13, 2013; revised April 25, 2014; accepted May 20, 2014; published OnlineFirst June 3, 2014.

References

1. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, et al. Cancer treatment and survivorship statistics. CA Cancer J Clin 2012;62:220–41.
2. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006;354:270–82.
3. Zhou Z, Qiao JX, Shetty A, Wu G, Huang Y, Davidson NE, et al. Regulation of estrogen receptor signaling in breast carcinogenesis and breast cancer therapy. Cell Mol Life Sci 2013. [Epub ahead of print].
4. Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol 2010;204:105–14.
5. Mizukami Y. *In vivo* functions of GPR30/GPER-1, a membrane receptor for estrogen: from discovery to functions *in vivo*. Endocr J 2010;57:101–7.
6. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, et al. G protein coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res 2007;7:1859–66.
7. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. EMBO J 2009;28:523–32.
8. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat Chem Biol 2006;2:207–12.
9. Lappano R, Rosano C, Santolla MF, Pupo M, De Francesco EM, De Marco P, et al. Two novel GPER agonists induce gene expression changes and growth effects in cancer cells. Curr Cancer Drug Targets 2012;12:531–42.
10. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, et al. *In vivo* effects of a GPR30 antagonist. Nat Chem Biol 2009;5:421–7.
11. Lappano R, Santolla MF, Pupo M, Sinicropi MS, Caruso A, Rosano C, et al. MIBE acts as antagonist ligand of both estrogen receptor α and GPER in breast cancer cells. Breast Cancer Res 2012;14:R12.
12. Lappano R, Rosano C, De Marco P, De Francesco EM, Pezzi V, Maggiolini M. Estriol acts as a GPR30 antagonist in estrogen receptor-negative breast cancer cells. Mol Cell Endocrinol 2010;320:162–70.
13. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 2000;14:1649–60.
14. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 2005;146:624–32.
15. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 2005;307:1625–30.
16. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, et al. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. Clin Cancer Res 2006;12:6359–66.
17. Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, et al. GPR30: a novel indicator of poor survival for endometrial carcinoma. Am J Obstet Gynecol 2007;196:386.e1–9; discussion 386.e9–11.
18. Smith HO, Arias-Pulido H, Kuo DY, Howard T, Qualls CR, Lee SJ, et al. GPR30 predicts poor survival for ovarian cancer. Gynecol Oncol 2009;114:465–71.
19. Ignatov A, Ignatov T, Weissenborn C, Eggemann H, Bischoff J, Semczuk A, et al. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. Breast Cancer Res Treat 2011;128:457–66.
20. Mo Z, Liu M, Yang F, Luo H, Li Z, Tu G, et al. GPR30 as an initiator of tamoxifen resistance in hormone-dependent breast cancer. Breast Cancer Res 2013;15:R114.
21. Madeo A, Maggiolini M. Nuclear alternate estrogen receptor GPR30 mediates 17b-estradiol-induced gene expression and migration in breast cancer associated fibroblasts. Cancer Res 2010;70:6036–46.
22. Gomes FG, Nedel F, Alves AM, Nör JE, Tarquinio SB. Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. Life Sci 2013;92:101–7.
23. Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. Crit Rev Oncol Hematol 2007;62:179–213.
24. Ferrara N. VEGF as a therapeutic target in cancer. Oncology 2005;69 Suppl 3:11–6.
25. Sengupta K, Banerjee S, Saxena N, Banerjee SK. Estradiol-induced vascular endothelial growth factor-A expression in breast tumor cells is biphasic and regulated by estrogen receptor-α dependent pathway. Int J Oncol 2003;22:609–14.
26. Stoner M, Wormke M, Saville B, Samudio I, Qin C, Abdelrahim M, et al. Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor α and SP proteins. Oncogene 2004;23:1052–63.
27. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604–1.
28. Sudhagar S, Sathya S, Lakshmi BS. Rapid non-genomic signalling by 17β-oestradiol through c-Src involves mTOR-dependent expression of HIF-1α in breast cancer cells. Br J Cancer 2011;105:953–60.
29. De Francesco EM, Lappano R, Santolla MF, Marsico S, Caruso A, Maggiolini M. HIF-1α/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). Breast Cancer Res 2013;15:R64.
30. Gerdes MJ, Myakishev M, Frost NA, Rishi V, Moitra J, Acharya A, et al. Activator protein activity regulates epithelial tumor cell identity. Cancer Res 2006;66:7578–88.
31. Albanito L, Sisci D, Aquila S, Brunelli E, Vivacqua A, Madeo A, et al. Epidermal growth factor induces G protein-coupled receptor 30 expression in estrogen receptor-negative breast cancer. Endocrinology 2008;149:3799–08.
32. da Silva BB, Lopes-Costa PV, dos Santos AR, de Sousa-Júnior EC, Alencar AP, Pires CG, et al. Comparison of three vascular endothelial markers in the evaluation of microvessel density in breast cancer. Eur J Gynaecol Oncol 2009;30:285–88.
33. Rafikova O, Rafikov R, Kumar S, Sharma S, Aggarwal S, Schneider F, et al. Bosentan inhibits oxidative and nitrosative stress and rescues occlusive pulmonary hypertension. Free Radic Biol Med 2013;56:28–43.
34. Staton CA, Reed MW, Brown NJ. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 2009;90:195–21.
35. Losordo DW, Isner JM. Estrogen and angiogenesis: a review. Arterioscler Thromb Vasc Biol 2001;21:6–12.
36. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005;438:967–74.
37. Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF, et al. Expression of vascular permeability factor (vascular endothelial

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

growth factor) and its receptors in breast cancer. Hum Pathol 1995;26:86–91.
38. Borgstrom P, Gold DP, Hillan KJ, Ferrara N. Importance of VEGF for breast cancer angiogenesis *in vivo*: implications from intravital microscopy of combination treatments with an anti-VEGF neutralizing monoclonal antibody and doxorubicin. Anticancer Res 1999; 19:4203–14.
39. Hyder S, Nawaz Z, Chiappetta C, Stancel G. Identification of functional estrogen response elements in the gene coding for the potent vascular endothelial growth factor. Cancer Res 2000;60:3183–90.
40. Hyder SM, Stancel GM, Chiappetta C, Murthy L, Boettger-Tong HL, Makela S. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Res 1996;56:3954–60.
41. Adams J, Carder PJ, Downey S, Forbes MA, MacLennan K, Allgar V, et al. Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissues VEGF and microvessel density and effects of tamoxifen. Cancer Res 2000;60:2898–05.
42. Smith HO, Stephens ND, Qualls CR, Fligelman T, Wang T, Lin CY, et al. The clinical significance of inflammatory cytokines in primary cell culture in endometrial carcinoma. Mol Oncol 2013;7:41–54.
43. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 2010;29:625–34.
44. Dery MA, Michaud MD, Richard DE. Hypoxia-inducible factor 1, regulation by hypoxic and non-hypoxic activators. Int J Biochem Cell Biol 2005;37:535–40.
45. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, et al. Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541–5.
46. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006;6: 392–01.
47. Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 2006,5:1597–601.
48. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–37.
49. Zhang HT, Craft P, Scott PA, Ziche M, Weich HA, Harris AL, et al. Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. J Natl Cancer Inst 1995;87:213–19.
50. Xie B, Tam NN, Tsao SW, Wong YC. Co-expression of vascular endothelial growth factor (VEGF) and its receptors (flk-1 and flt-1) in hormone-induced mammary cancer in the Noble rat. Br J Cancer 1999;81:1335–43.

Published OnlineFirst June 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3590

Cancer Research

GPER Mediates Activation of HIF1α/VEGF Signaling by Estrogens

Ernestina Marianna De Francesco, Michele Pellegrino, Maria Francesca Santolla, et al.

Cancer Res 2014;74:4053-4064. Published OnlineFirst June 3, 2014.

REPORT

Cell Cycle 13:4, 553–564; February 15, 2014; © 2014 Landes Bioscience

Evidences that estrogen receptor α interferes with adiponectin effects on breast cancer cell growth

Loredana Mauro[1,*], Michele Pellegrino[1], Francesca De Amicis[1,2], Emilia Ricchio[1], Francesca Giordano[1], Pietro Rizza[1], Stefania Catalano[1,2], Daniela Bonofiglio[1,2], Diego Sisci[1,2], Maria Luisa Panno[1], and Sebastiano Andò[1,2,*]

[1]Department of Pharmacy, Health, and Nutritional Sciences; University of Calabria; Cosenza, Italy; [2]Centro Sanitario; University of Calabria; Cosenza, Italy

Keywords: breast cancer, adiponectin, estrogen receptor alpha, cell proliferation, MAPK, IGF-IR, AdipoR1, APPL1

Abbreviations: ERα, estrogen receptor alpha; AdipoR1, adiponectin receptor 1; IGF-IR, insulin-like growth factor I receptor; EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKA, cAMP-dependent protein kinase; E_2, 17β-estradiol

Adiponectin, the most abundant protein secreted by adipose tissue, exhibits insulin-sensitizing, anti-inflammatory, antiatherogenic, and antiproliferative properties. In addition, it appears to play an important role also in the development and progression of several obesity-related malignancies, including breast cancer.

Here, we demonstrated that adiponectin induces a dichotomic effect on breast cancer growth. Indeed, it stimulates growth in ERα+ MCF-7 cells while inhibiting proliferation of ERα− MDA-MB-231 cells. Notably, only in MCF-7 cells adiponectin exposure exerts a rapid activation of MAPK phosphorylation, which is markedly reduced when knockdown of the ERα gene occurred. In addition, adiponectin induces rapid IGF-IR phosphorylation in MCF-7 cells, and the use of ERα siRNA prevents this effect. Moreover, MAPK activation induced by adiponectin was reversed by IGF-IR siRNA. Coimmunoprecipitation studies show the existence of a multiprotein complex involving AdipoR1, APPL1, ERα, IGF-IR, and c-Src that is responsible for MAPK signaling activation in ERα+ positive breast cancer cells. It is well known that in addition to the rapid effects through non-genomic mechanisms, ERα also mediates nuclear genomic actions. In this concern, we demonstrated that adiponectin is able to transactivate ERα in MCF-7 cells. We showed the classical features of ERα transactivation: nuclear localization, downregulation of mRNA and protein levels, and upregulation of estrogen-dependent genes. Thus, our study clarifies the molecular mechanism through which adiponectin modulates breast cancer cell growth, providing evidences on the cell-type dependency of adiponectin action in relationship to ERα status.

Introduction

Obesity is associated with an increase in white adipose tissue that greatly alters the local and systemic secretion of biologically active polypeptides, known as adipocytokines.[1] Currently more than 50 different adipocytokines have been identified, which mainly regulate energy metabolism, but also have several pathophysiological functions.[2] Of these, adiponectin is a 244 amino acid-long polypeptide that represents the most abundant adipose tissue-derived hormone. It has a protective role against obesity-related disorders, including metabolic syndrome, type-2 diabetes, and cardiovascular disease.[3] Unlike most of the other adipokines, serum adiponectin is inversely correlated with body mass index (BMI).[4,5] Circulating adiponectin levels are reduced in obesity and type 2 diabetes,[6] and mice lacking adiponectin develop insulin resistance, glucose intolerance, hyperglycemia, and hypertension, all characteristics of metabolic syndrome.[7,8] A speculative explanation of the reduced adiponectin levels in obesity may be sustained by the enhanced production of cytokines that occurred in such condition, and may contribute to the downregulatory effect on adiponectin secretion by adipose tissue.[9] Another potential mechanism indicates a negative feedback of adiponectin on its own production and probably on the expression of its receptors during the development of obesity.[10] Adiponectin is synthesized as a single subunit that undergoes oligomerization to form trimers, hexamers, and multimers before secretion. In serum, adiponectin exists in its full-length form (fAdiponectin) or as a proteolytic fragment that corresponds to the globular domain of the protein (gAdiponectin) with enhanced potency.[11] The cellular functions of adiponectin are mediated through the adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) and T-cadherin.[3] AdipoR1 and AdipoR2 are integral membrane proteins containing 7 transmembrane domains but are structurally and functionally

*Correspondence to: Loredana Mauro; Email: doclollo@yahoo.com; Sebastiano Andò; Email: sebastiano.ando@unical.it
Submitted: 09/19/2013; Revised: 12/06/2013; Accepted: 12/06/2013; Published Online: 12/06/2013
http://dx.doi.org/10.4161/cc.27455

distinct from G protein-coupled receptors.[12] AdipoR1 presents high affinity for gAdiponectin and low affinity for fAdiponectin, and it is expressed ubiquitously but abundantly in skeletal muscle and endothelial cells. AdipoR2 has intermediate affinity for both forms of adiponectin and is predominantly expressed in the liver.[13] It has been demonstrated that the globular form of adiponectin binds to AdipoR1,[12] which, in turn, through the adaptor protein APPL1 interacting with the intracellular N terminus of AdipoR1, induces activation of MAPK through Src pathway.[14,15] APPL1, which contains a pleckstrin homology domain, a phosphotyrosine-binding domain, and a leucin zipper motif, has emerged as an important element in AdipoR1/R2 signaling.[14]

Recent experimental and clinical investigations suggested that low levels of plasma adiponectin are associated with an increased risk for obesity-related cancers, such as colon, prostate, endometrial, and breast cancer.[10,16,17] The pathogenesis of breast cancer is largely dependent on interactions between malignant cells and components of the breast microenvironment. The malignant cell phenotype is regulated not only by autonomous signals originating from cancer cells, but also by the effects of the surrounding stromal cells, which influence mammary epithelial cell growth and differentiation.[2] Most important, some studies have suggested that breast tumors arising in patients with hypoadiponectinemia may have a more aggressive phenotype (large tumor size, high histological grade, estrogen receptor negativity, and increased metastasis).[17,18] Particularly, in the mammary gland, epithelial cells are exposed to both circulating and locally produced adiponectin from adjacent adipocytes. The close association between mammary epithelial cells and adipocytes may favor a more direct action of adipokines on that tissue.[19] Recently it has been reported that low circulating adiponectin levels are associated with a higher risk of breast cancer development, and that this association is independent of age, BMI, and hormonal and reproductive factors.[19] Consistent with these evidences, it has been demonstrated that adiponectin mediates an antiproliferative effect in human ERα-negative MDA-MB-231 breast cancer cells through the regulation of genes involved in cell cycle, such as p53, Bax, Bcl-2, c-myc, and cyclin D1.[20-22] On the other hand, conflicting observations have been reported on the effects elicited by adiponectin in ERα-positive MCF-7 cells. For instance, some authors evidenced significant anti-proliferative effects;[19,22-24] other studies didn't show any noticeable effect;[20,25] while some other studies demonstrated a stimulatory effect on the growth of this cell line.[26,27]

Thus, stemming from these controversial data, the aim of the present study was to investigate whether the ERα expression influences the response to adiponectin in breast cancer growth.

Our data demonstrated that in ERα-positive breast cancer cells, adiponectin is able to activate ERα at both non-genomic and genomic level, stimulating breast cancer growth.

Results

Adiponectin induces divergent effects on breast cancer cell proliferation

We first investigated the effect of adiponectin (1 and 5 μg/ml) on cell proliferation in both estrogen receptor α-negative (ERα–) as well as positive (ERα+) breast cancer cell lines, by MTT growth assays. After 3 d treatment, adiponectin inhibited cell proliferation of ERα– MDA-MB-231 and SKBR-3 cells, whereas it induced growth in MCF-7 and T47D cells (**Fig. 1A**), which express high levels of ERα.[28]

The same dichotomic pattern has been reproduced in anchorage-independent growth assays, using both ERα+ and ERα– breast cancer cells (**Fig. 1B**).

We then performed 3-dimensional MCF-7 cell cultures, which closely mimic some in vivo biologic features of tumors.[29] Our results demonstrated that adiponectin treatment enhanced cell–cell adhesion (**Fig. 1C**) as well as increased cell growth (**Fig. 1D**) compared with untreated cells.

In order to investigate the role of ERα in modulating the effect of adiponectin on cell proliferation, in MCF-7 cells ERα was knocked down by siRNA or abrogated by the potent and specific antiestrogen ICI 182780. In these conditions the adiponectin-induced cell proliferation was completely reversed (**Fig. 1E**).

These results address how adiponectin may affect breast cancer growth through the involvement of ERα.

Effect of adiponectin on ERα non-genomic signal

The biological actions of ERα are mediated by non-genomic action outside of the nuclear compartment and by genomic effects via nuclear ERs.[30] The non-genomic effects lead to the rapid activation of the MAPK signaling pathway. Both the genomic and non-genomic actions of ERα play pivotal roles in ERα-induced cancer cell proliferation and survival.

Thus, we investigated the possible cross-talk existing between membrane ERα transductional pathways and adiponectin receptor, since both signaling appear to converge to MAPK cascade. Indeed, it has been demonstrated that AdipoR1 binds to the adaptor protein APPL1[14] and induces activation of MAPK through Src pathway.[15] For instance, c-Src is also an initial and integral crossroad of different membrane signaling events mediated by the ERα as well as by its cross-talk with growth factors. Thus, we found it reasonable to verify whether AdipoR1/APPL1 may interact with other membrane signaling involved in breast cancer cell growth and progression. We demonstrated for the first time how, in basal conditions, APPL1 coimmunoprecipitated with AdipoR1, ERα, IGF-IR, and c-Src, but not with EGF-R at both 15 min (**Fig. 2A**) and 48 h (**Fig. 2B**). These interactions appeared enhanced by adiponectin exposure (**Fig. 2A and B**).

Figure 2C shows the in vitro effect of the protein complex on Src kinase activity. MCF-7 cell lysates were immunoprecipitated with an anti-APPL1 antibody, and the Src kinase activity was measured using the exogenous acid-treated enolase as substrate in the absence and in the presence of adiponectin after 15 min of exposure. The results provide evidence that adiponectin activates c-Src, as evidenced by both the autophosphorylation of c-Src and the concomitant phosphorylation of enolase. Furthermore, we observed an increase of IGF-IR phosphorylation after 15 and 30 min of adiponectin treatment, which was reduced after knockdown of ERα (**Fig. 2D**).

In MCF-7 cells we observed a rapid activation of MAPK by adiponectin (**Fig. 2E**). To determine the mechanism through

which AdipoR1 stimulates MAPK, we employed a panel of RNA silencers targeting ERα, IGF-IR, and APPL1 (**Fig. 2F**), or selective inhibitor targeting Src family kinase or PKA. We observed that MAPK activity was abrogated in the presence of all these agents (**Fig. 2E**), suggesting that MAPK cascade upon adiponectin exposure requires APPL1, ERα, IGF-IR, c-Src, and PKA activity.

Finally, in the presence of APPL1 silencer, AdipoR1 was no longer associated with ERα, IGF-IR, and c-Src (data not shown).

The role of ERα membrane signal in the above reported membrane complex emerges from the evidence that in ERα–MDA-MB-231 cells, adiponectin was no longer able to induce MAPK phosphorylation (**Fig. 3A**). However, when these cells were transfected with a membrane ERα (mERα) construct, which consists solely of the AF-2/ligand binding domain of ERα, adiponectin short exposure was able to upregulate MAPK activity (**Fig. 3B**).

Adiponectin transactivates ERα through MAPK signaling

Several studies in recent years suggest that membrane-initiated signaling can converge into genomic events, leading to more long-term consequences. For instance, in MCF-7 breast cancer cells adiponectin revealed an induced MAPK activation still after 48 h treatment (**Fig. 4A**). Since MAPK is involved in enhancing ERα functional activation in a ligand-independent manner, we wondered whether adiponectin was able to modulate ERα transactivation.

In MCF-7 cells exposed to adiponectin for 48 h, a significant activation of the estrogen-responsive gene XETL was observed (**Fig. 4B**). Similar results were reproduced in ER-negative HeLa

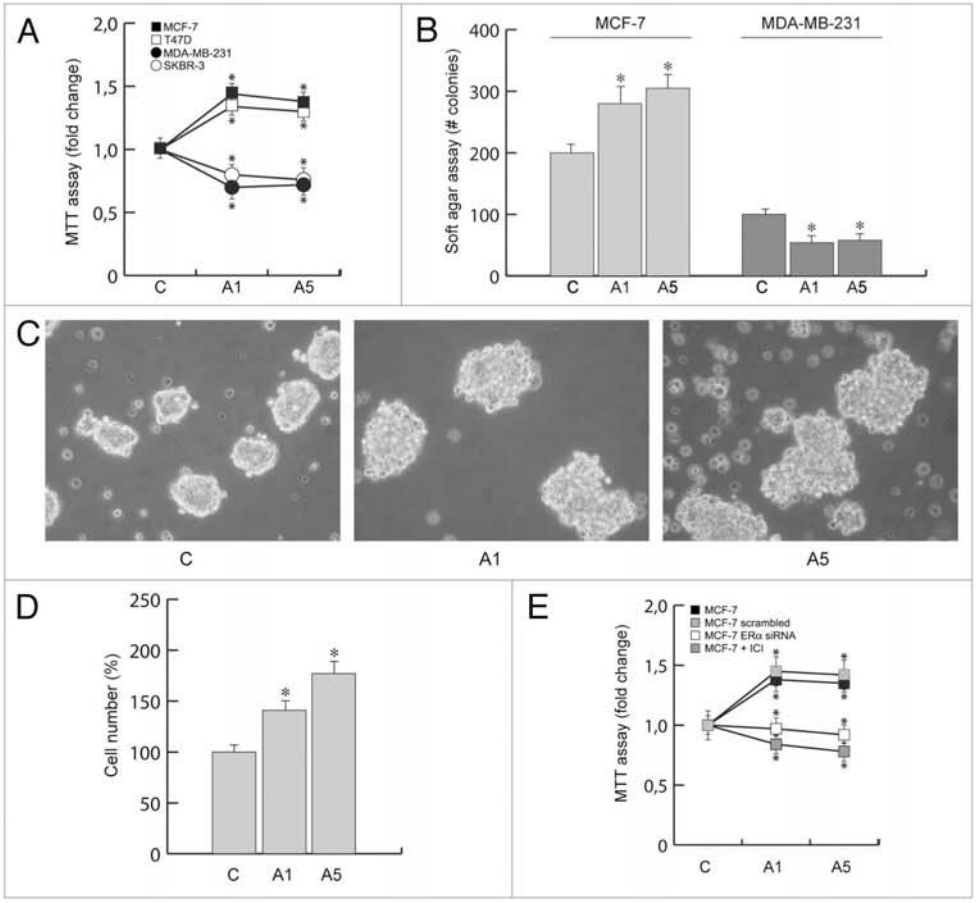

Figure 1. Effects of adiponectin on breast cancer cell growth. (**A**) MTT growth assays in MCF-7, T47D, MDA-MB-231, and SKBR-3 cells treated with vehicle (**C**) or adiponectin 1 (A1) or 5 μg/ml (A5) for 72 h. Cell proliferation is expressed as fold change ± SD relative to vehicle-treated cells and is representative of 3 different experiments each performed in triplicate. (**B**) Soft agar growth assay in MCF-7 and MDA-MB-231 cells plated in 0.35% agarose and treated as indicated above. After 14 d of growth colonies >50 μm diameter were counted. (**C**) MCF-7 3-dimensional cultures were untreated or treated as indicated for 48 h and then photographed under phase-contrast microscopy. (**D**) Cell numbers obtained from 3-dimensional spheroids in MCF-7 cells treated as indicated for 48 h. (**E**) MCF-7 cells exposed to ICI 182 780, or transfected in suspension with 30 nM siRNAs/well (ERα siRNA or a scrambled siRNA for control samples), were treated with vehicle (**C**) or A1 or A5 for 72 h before testing cell viability using MTT assay. Results are expressed as fold change ± SD relative to vehicle-treated cells and are representative of 3 different experiments, each performed in triplicate. *$P < 0.05$ compared with vehicle.

cells ectopically expressing ERα and transfected with XETL (**Fig. 4B**). It is worth noting that the specific anti-estrogen ICI 182780 efficiently antagonized the stimulatory effect of adiponectin on ERα transactivation in both cell types (data not shown).

In the presence of the MAPK inhibitor PD98059, the direct capability of adiponectin in transactivating ERα was completely lost. When we tested different inhibitors of signaling converging on MAPK activation, we observed how PKA and c-Src inhibitors, H89 and PP2, respectively, were able to prevent adiponectin-induced ERα transactivation (**Fig. 4C**). Moreover, knocking down of IGF-IR exerted similar effects (**Fig. 4D**). These results address MAPK signaling as crucial in modulating ERα transactivation upon adiponectin exposure.

In addition, we also studied which functional domain was involved in ERα transactivation by adiponectin. To this aim, HeLa cells were cotransfected with the XETL reporter gene and plasmids codifying for AF-1 or AF-2 domain (**Fig. 5A**). The treatment with adiponectin induced an increased transcriptional activation only in transfected cells bearing the plasmid codifying for AF-1 domain (**Fig. 5B**). These data demonstrate that the N-terminal AF-1 domain is essential in mediating the adiponectin response. Moreover, the activation of ERα occurred at the genomic level, as demonstrated by trasfecting HeLa cells

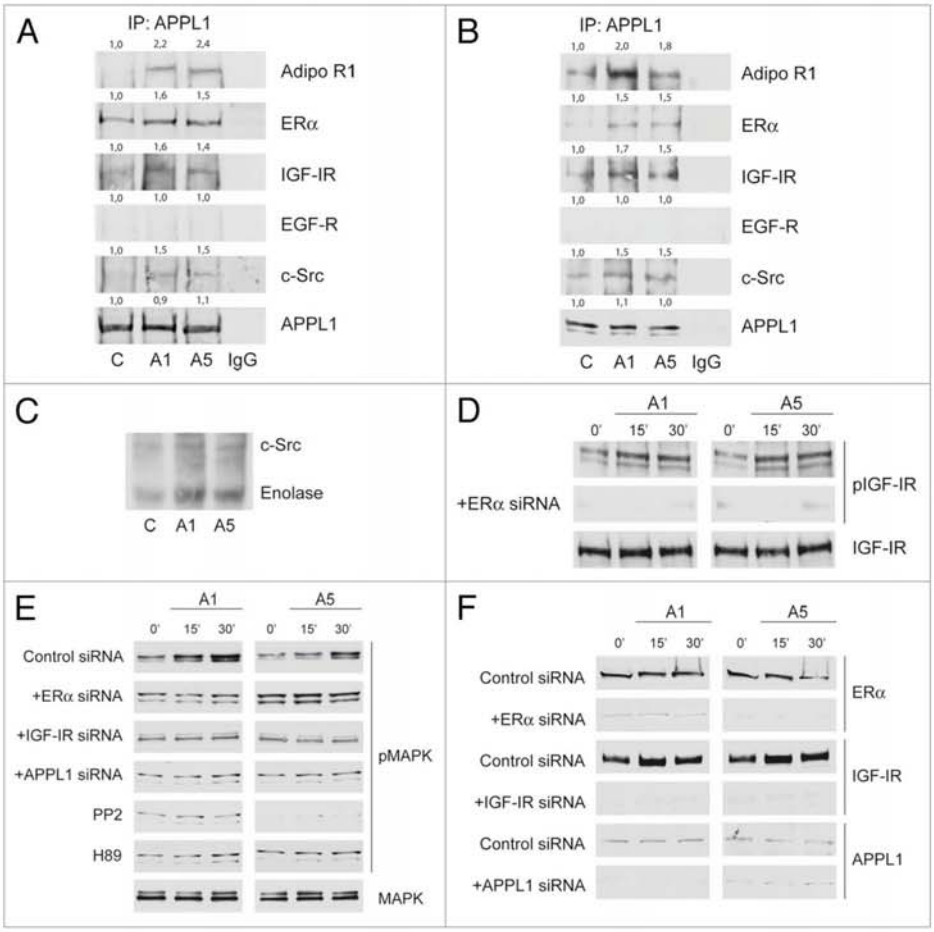

Figure 2. Adiponectin induces MAPK activation through the formation of a multimeric protein complex. Five hundred micrograms of protein lysates from MCF-7 cells, untreated (**C**) or treated with A1 or A5 for 15 min (**A**) or 48 h (**B**), were immunoprecipitated with an APPL1 antibody and then blotted with the indicated antibodies on the right. To verify equal loading, the membrane was probed with anti-APPL1 antibody. Normal rabbit IgG was used as a negative control to precipitate A5-treated samples. Numbers on top of the blots represent the average fold change vs. untreated cells. One of 3 similar experiments is presented. (**C**) MCF-7 cells, treated with adiponectin for 15 min, were lysed and immunoprecipitated with an anti-APPL1 antibody/protein A/G complex and assayed for c-Src-kinase activity using acid-treated enolase as described in "Materials and Methods". These results are representative of 3 independent experiments. c-Src and enolase position is indicated. (**D**) pIGF-IR^{Tyr1131} levels in MCF-7 cells treated with vehicle (–) or A1 and A5 as reported in absence or presence of ERα siRNA. (**E**) Total cellular proteins were isolated from MCF-7 cells in the absence or presence of ERα, IGF-IR, or APPL1 siRNA or pre-treated with PP2 or H89 and treated with A1 and A5. pMAPK, levels were evaluated by immunoblotting. Total MAPK was used as a loading control. (**F**) ERα, IGF-IR, and APPL1 levels were shown as control of silencing. Western blotting showed are representative of 3 different experiments.

with the plasmid HE241G, encoding ERα lacking the nuclear localization sequence (**Fig. 5B**).

The involvement of AF-1 domain in the activation of ERα by adiponectin was confirmed cotransfecting HeLa cells with XETL and with either HEGO or different plasmids in which ERα was mutated in specific phosphorylation sites, such as Ser-104/106/118A-ER or S118A-ER or S167A-ER (**Fig. 5C**), which are effectors of phosphorylative signaling. As shown in **Figure 5D**, in transfectants with either Ser-104/106/118A-ER or S118 mutants, adiponectin was no longer able to elicit any substantial activation on ERE luciferase signal as compared with the cells bearing wild-type ERα. In contrast, the activation still persisted in cells transfected with S167 mutant (**Fig. 5D**). Western blotting analysis confirmed how adiponectin enhanced the phosphorylation of ERα at Ser118, while it was unable to affect activation at Ser167 (**Fig. 5E**). The biological correlates of these events reproduce the classic features of ERα transactivation in breast cancer cells. In MCF-7 cells cultured in serum deprivation conditions for 96 h, ERα immunoreactivity was no longer detectable in the control, whereas treatment with adiponectin for 24 h induced a strong ERα immunoreactivity in the nuclear compartment (**Fig. 6A**), which was abrogated in the presence of MAPK inhibitor PD98059 (data not shown). These results show that adiponectin mimics the effect of estradiol on ERα compartmentalization in breast cancer cells. In addition, in MCF-7 cells, we observed a significant downregulation of ERα mRNA and protein levels after adiponectin treatment (**Fig. 6B and C**), which is a typical hallmark of the receptor activation by an agonist. Moreover, adiponectin was able to increase the expression of classic estrogen-dependent genes, such as Catepsin D and pS2 (**Fig. 6D**), providing further evidence for the ability of this adipokine to activate ERα.

Evidence for the contribution of the multiprotein complex in adiponectin-induced MCF-7 cell growth

Finally, we evaluated the involvement of the multiprotein complex in mediating adiponectin-induced MCF-7 cell growth. When we knocked down the components of the multiprotein complex (ERα, IGF-IR, and APPL1) or inhibited its signaling cascade, through PP2, H89, and PD98059 (inhibitors of c-Src, PKA, and MAPK, respectively), the stimulatory role of adiponectin on MCF-7 cell growth was completely abrogated (**Fig. 7**).

Discussion

Accumulating evidences have addressed how adipose tissue can act as an effector organ that influences breast cancer risk, tumor behavior, and clinical outcome.[31]

The main underlying mechanisms that link obesity to breast cancer development and progression include: (1) abnormalities of insulin resistance and the IGF-I system deregulation; (2) the impact of adiposity on the biosynthesis and bioavailability of endogenous sex hormones; (3) obesity-induced low-grade chronic systemic inflammation; and (4) alterations in the levels of adipocyte-derived factors.[32] Indeed, adipose tissue is now widely considered to be an active endocrine organ, secreting several bioactive adipokines, including adiponectin, that exert distinct metabolic functions.[33] Adiponectin plays a protective function against obesity-related disorders and the metabolic syndrome, particularly in the pathogenesis of type II diabetes and cardiovascular disease.[34] Beyond these metabolic effects, adiponectin deficiency, commonly observed in obesity, may contribute to the natural history of several malignancies, such as breast cancer.[18] In particular, low adiponectin levels have been strongly associated with an increased breast cancer risk, and patients with reduced adiponectin levels develop a biologically aggressive phenotype independently of estrogen receptor status,[16] although inconclusive results have been published in the latter concern. For instance, several data point toward cell line-dependent effects.[35] Adiponectin has been shown to inhibit the growth of ERα– normal MCF-10A human mammary epithelial cells,[25] as well as cancerous MDA-MB-231[20,27,36] and SKBR-3[22] cells. This anti-proliferative effect involves an enhanced expression of Bax, Bcl-2, p53, c-myc, and reduced level of cyclin D1.[37]

However, in ERα+ MCF-7 and T47D cells, adiponectin appears to stimulate[26,27] or to inhibit cell growth,[19,22-24] or to play no noticeable effect.[20,25] Possible explanations of the different cellular responses may be different content of ERα, differences in culture conditions, specific adiponectin isoform used, incubation time, or dosage.

Here, we elucidated the complex mechanisms involved in adiponectin response in breast cancer growth in dependency on ERα status. Particularly, we demonstrated that MAPK activation, induced by adiponectin/ERα-mediated effect, produces MCF-7 cell proliferation, and it represents the discriminator factor determining the opposite effect induced by adiponectin in ERα+ and ERα– breast cancer cells.

For instance, adiponectin inhibits the growth of ERα– breast cancer cells, whereas it induces proliferation in ERα+ cells.

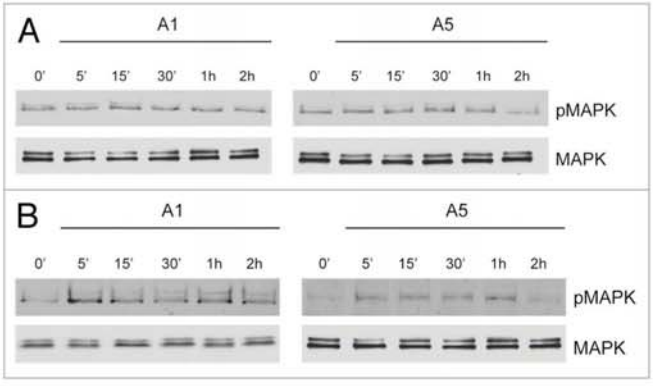

Figure 3. Time course of MAPK activation upon adiponectin exposure in MDA-MB-231 cells ectopically expressing membrane ERα. MDA-MB-231 (**A**) and MDA-MB-231 transfected with a plasmid codifying for membrane ERα (**B**) were serum-starved for 24 h followed by treatment with adiponectin 1 or 5 μg/ml for the indicated times. Western blots show the phosphorylation status of MAPK. Total MAPK is used as a loading control. One of 3 similar experiments is presented.

The evidence that this dichotomic effect is dependent on the expression of ERα emerges from the observation that in the presence of its knocking down, adiponectin induced inhibition of MCF-7 cell growth. Thus, we asked through which molecular mechanisms ERα may interfere with adiponectin effect on breast cancer cell growth.

It has been described that a fraction of ERα is extranuclear and exists as cytoplasmatic/membrane-bound population that mediates rapid "non-genomic" events, leading to cell morphologic changes and inhibition of apoptosis.[30,38] According to this function, cytoplasmatic ERα can physically interact with different signaling molecules, including IGF-IR[38] and c-Src.[39] Recent findings demonstrated that AdipoR1, through its most important adaptor protein APPL1, activated c-Src/MAPK pathway as specific effector of adiponectin signaling.[15]

Thus, APPL1 appears to be a key intracellular mediator of most of adiponectin responses reported to date. Activation of MAPK cascade is essential for cell cycle initiation and plays a key role in the control of cell proliferation, differentiation, and survival,[40] hypothesizing that adiponectin might act as a growth factor. MAPK serves as a point of convergence for diverse membrane signal inputs. All this has given the rationale to ascertain the existence of a mechanistic link existing between AdipoR1/APPL1 and other ERα driven membrane signaling. To date, nearly 14 proteins have been reported to associate with APPL1 in various types of cells, and they could be categorized into 3 different groups: membrane receptors, signaling proteins, and others.[41] On the basis of coimmunoprecipitation assays, we demonstrated that AdipoR1 and APPL1 interact with ERα, c-Src, and IGF-IR, all converging into MAPK activation. Indeed, the latter event was completely abrogated in the presence of ERα, IGF-IR, and APPL1 siRNA or pharmacological inhibitor of c-Src, PP2. The role of c-Src in maintaining protein complex formation is sustained by evidence demonstrating that, in MCF-7 cells, it allows ERα interaction with IGF-IR, exhibiting a docking site for ERα membrane

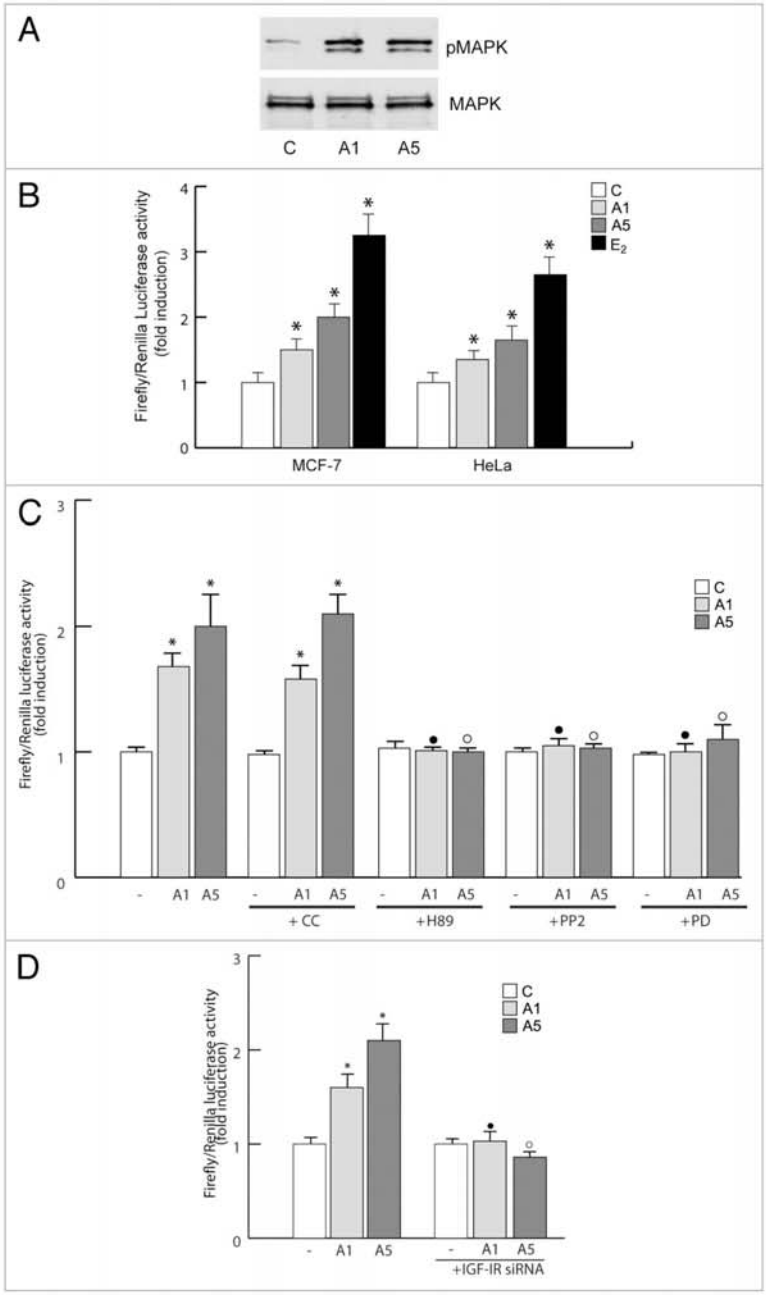

Figure 4. Effects of adiponectin on ERα transactivation. (**A**) Total cellular proteins were isolated from MCF-7 cells treated with A1 and A5 for 48 h. pMAPK, levels were evaluated by immunoblotting. Total MAPK was used as a loading control. (**B**) MCF-7 cells were transfected with the luciferase reporter plasmid XETL. HeLa cells were cotransfected with XETL and HEGO plasmids. The cells were untreated or treated for 48 h with A1 and A5 or 100 nM E_2 used as positive control. (**C**) MCF-7 cells were transfected with the luciferase reporter plasmid XETL. The cells were untreated or treated for 48 h with A1 and A5 or in combination with Compound C (CC), H89, PP2, or PD98059. *$P < 0.05$ compared with control (–); •$P < 0.05$ compared with A1; ○$P < 0.05$ compared with A5. (**D**) MCF-7 cells were transfected with XETL plasmid in the absence or presence of IGF-IR siRNA and treated with adiponectin for 48 h. *$P < 0.05$ compared with control (–). The values represent the means ± SD of 3 different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections.

association.[38,39] Moreover, our results demonstrated that short exposure to adiponectin was able to rapidly enhance tyrosine phosphorylation of IGF-IR, which was no longer noticeable in the presence of ERα siRNA. On the basis of these findings, we hypothesize that adiponectin induces a linear pathway involving AdipoR1, ERα/IGF-IR/c-Src/MAPK.

The role of ERα in mediating the adiponectin-induced activation of MAPK in breast cancer cells emerges from the evidence that ectopical expression of membrane ERα in MDA-MB-231 cells was able to restore MAPK phosphorylation by adiponectin. This addresses how the presence of rapid ERα membrane signal is a prerequisite for adiponectin-induced MAPK activation.

In addition, membrane ERα, driving membrane signaling, may, in the long-term, induce a cascade of events converging in MAPK activation, which, in turn, transactivates ERα at genomic level.[30] In our study, we have provided the first evidence that adiponectin induces transactivation of ERα in MCF-7 cells. Indeed, it is worth observing that this occurred according to the classical features of ERα transactivation: nuclear localization, downregulation of mRNA and protein levels, and upregulation of estrogen-dependent genes.

Adiponectin was able to transactivate ERα in a ligand-independent manner, since only the AF-1 domain is essential in mediating the specific response through the phosphorylation of Ser118. Serine118 in the N terminus of human ERα is a well-studied phosphorylation site, and both rapid estrogens action and growth factors such as EGF and IGF-I, all converging in MAPK activation, result in phosphorylation of Ser118.[42] Indeed, our results demonstrated that upon adiponectin exposure, ERα transactivation

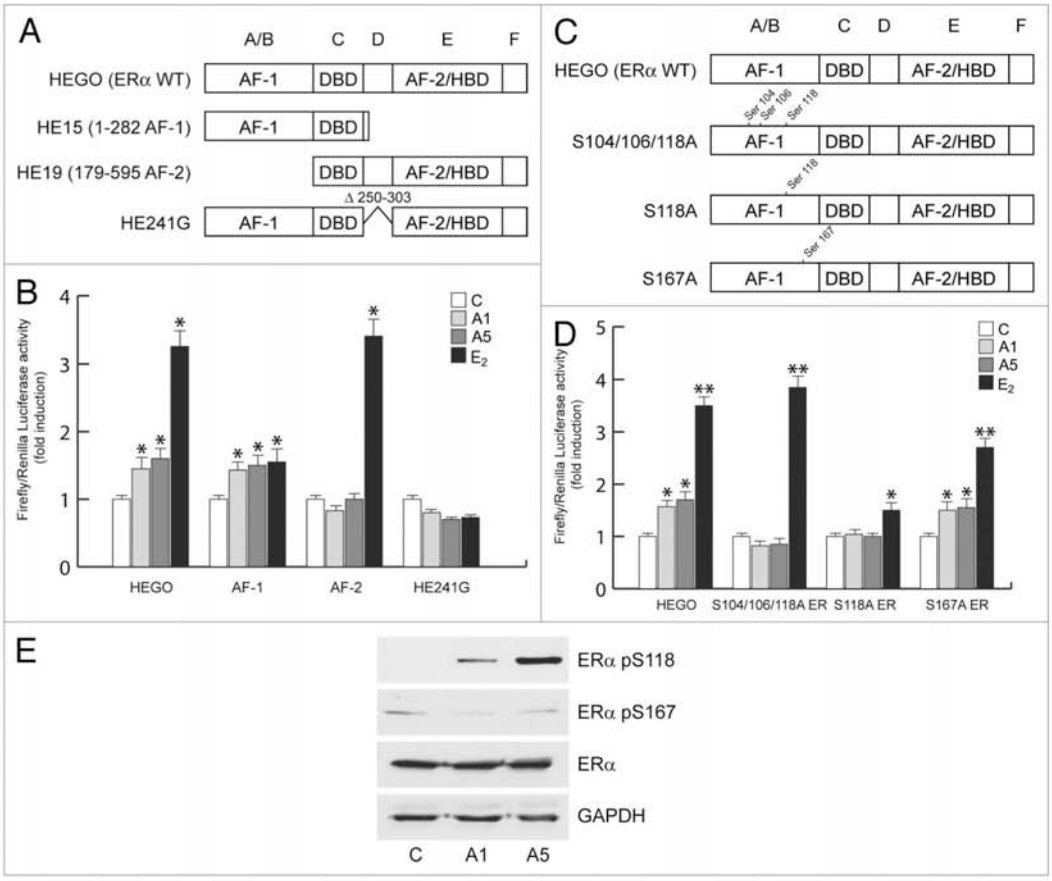

Figure 5. Effects of adiponectin on ERα functional domains. (**A and C**) Schematic illustration of ER constructs used for the experiments. HEGO is a wild-type ER-expressing vector that encodes a 595-amino acid protein. HE15-(1–282) contains AF-1 and the DNA binding domain (DBD). HE19-(179–95) contains DBD, and AF-2 domains. HE241G encodes a mutated ERα, which has the nuclear localization sequence deleted (250–303). ER plasmids mutated in serine residues 104, 106, 118, and 167 to Ala. (**B**) HeLa cells were transiently cotransfected with XETL and either HEGO or PSG5/HE15 (AF-1) or PSG5/HE19 (AF-2) or HE241G plasmids. (**D**) HeLa cells were transiently cotransfected with XETL and either HEGO or Ser-104/106/118A-ER or Ser-118A-ER or Ser-167A-ER. The cells were treated for 48 h in the absence (**C**) or in the presence of A1 and A5 or E_2 (100 nM) used as positive control. The values represent the means ± SD of 3 different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *$P < 0.05$, **$P < 0.01$ compared with (**C**). (**E**) Total extracts from cells treated with A1 or A5 for 30 min were analyzed for phosphorylation of serines 118 and 167 (pS118 and pS167) and expression of ERα by immunoblot analysis. GAPDH was used as a control for equal loading and transfer.

was abrogated in the presence of MAPK inhibitor PD98059. Also, PKA appears to play a role in the latter concern, since its inhibition abolishes adiponectin-stimulated MAPK activation.

In our model, we demonstrated that adiponectin via PKA signal influences MAPK pathway, thus inducing ERα transactivation. Indeed, both adiponectin-induced MAPK phosphorylation and ERα transactivation are completely abrogated in the presence of a specific PKA inhibitor, H89.

However, our results showed that adiponectin action on ERα transactivation was independent by phosphorylation of Ser167, targeted by PKA signaling, while it failed to induce ERα signaling in cells ectopically expressing ERα mutated in Ser118, usually phosphorylated by MAPK.

All these observations lead to the idea that adiponectin effects on ERα transactivation are based mostly on MAPK signaling, which is dependent on the formation of a multiprotein complex including AdipoR1/APPL1/c-Src/ ERα/IGF-IR. On the other hand, the activation of MAPK by PKA may occur through c-Src/ Rap1/B-Raf cascade.[43] Thus the adiponectin/AdipoR1/ERα axis, through c-Src, may converge on MAPK activation.

Adiponectin-induced transactivation of ERα appears not to involve another important effector signaling, such as AMPK, since it is not substantially affected by its pharmacological inhibition.

In conclusion, we provided evidence that the effects of adiponectin on breast cancer cell proliferation are dependent on ERα expression through both its non-genomic and genomic actions (**Fig. 8**).

Thus, our results suggest that ERα negatively interferes with the antiproliferative effect induced by adiponectin on breast cancer cell growth.

On the basis of these observations, it is rational to conclude that at the doses tested, adiponectin exerts an antiproliferative role only in ERα-negative breast cancer cells, wherein it may represent a promising pharmacological tool to be implemented in the novel adjuvant strategies adopted in breast cancer.

Materials and Methods

Cell culture

Cells utilized in the studies were obtained from American Type Culture Collection, where they were authenticated, stored according to supplier's instructions, and used within 4 mo after frozen aliquots resuscitations. Every 4 mo, cells were authenticated by single tandem repeat analysis at our Sequencing Core; morphology, doubling times, estrogen sensitivity, and mycoplasma negativity were tested (MycoAlert, Lonza).

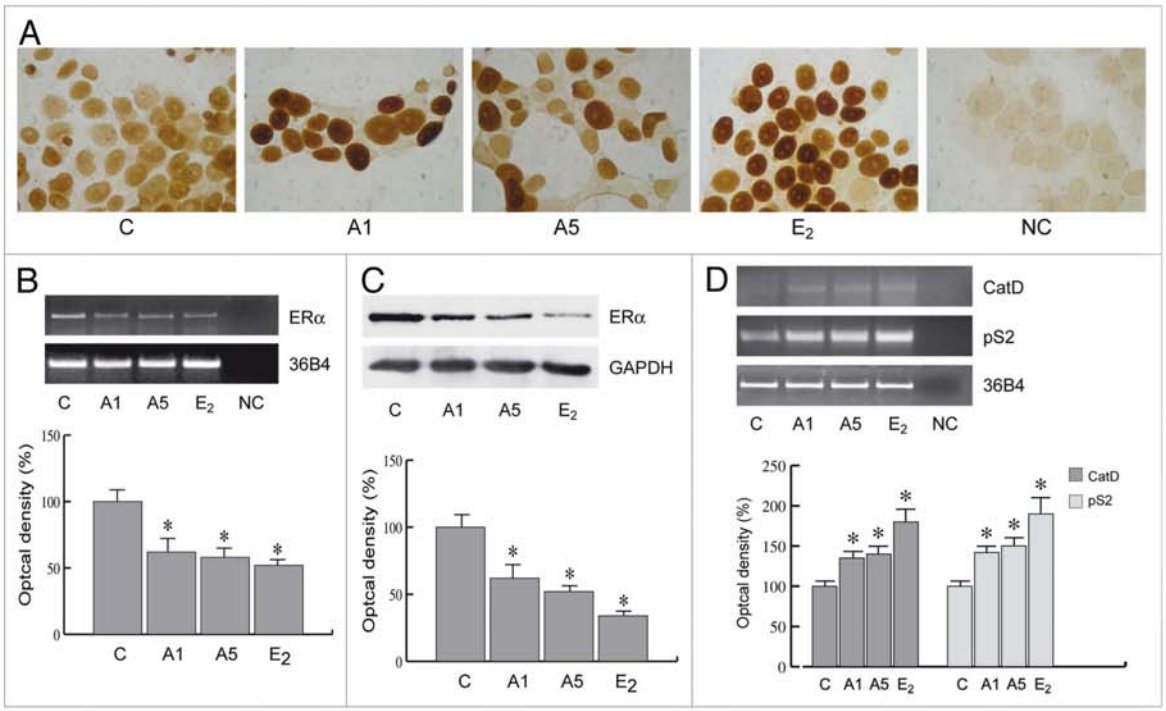

Figure 6. Adiponectin actions on cellular localization and expression of ERα. (**A**) MCF-7 cells were incubated in serum-free medium for 96 h and then treated with vehicle (**C**), A1, A5, or 100 nM E_2, used as positive control, for 24 h. No immunodetection was observed replacing the anti-ER antibody with an irrelevant mouse IgG (NC). Each experiment is representative of at least 10 tests. (**B**) RT-PCR of ERα mRNA. MCF-7 cells were stimulated for 48 h with A1 and A5 or E_2 (100 nM); 36B4 mRNA levels were determined as a control. (**C**) Immunoblot of ERα from MCF-7 cells treated as above; GAPDH serves as loading control. (**D**) RT-PCR of Catepsin D (CatD) and pS2 mRNA. MCF-7 cells were treated as reported. The histograms represent the mean ± SD of 3 separate experiments, in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *$P < 0.05$ vs. control.

MCF-7, T47D, and HeLa cells were cultured as described.[44] MDA-MB-231 were maintained in DMEM/F-12 containing 5% fetal bovine serum (Sigma). SKBR-3 breast cancer cells were cultured in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS). Before each experiment, cells were grown in phenol red-free media, for at least 24 h and then treated with 1 and 5 μg/ml recombinant human gAdiponectin/gAcrp30 (R&D Systems) as described.

Cell proliferation assays

MTT assays

After 3 d of treatment, cell proliferation was assessed by MTT assay as reported[45] and expressed as fold change relative to vehicle-treated cells.

Soft agar growth assays

Anchorage-independent growth assays were conducted as previously described.[46] Data represent 3 independent experiments performed in triplicate.

Three-dimensional spheroid culture and cell growth assays

For 3-dimensional cultures, MCF-7 cells plated on 2% agar-coated plates were untreated or treated with adiponectin 1 and 5 μg/ml. After 48 h, 3-dimensional cultures were photographed using a phase-contrast microscope (Olympus), and the cell numbers were evaluated as previously reported.[47]

RNA silencing

MCF-7 cells were transfected with RNA duplex of stealth siRNA targeted for the human ERα (SI02781401), IGF-IR (SI01074017), or APPL1 (GS26060) mRNA sequence or with a control siRNA (Qiagen, Milan, Italy) that does not match with any human mRNA, used as a control for non-sequence-specific effects. Cells were transfected using RNAiFect Transfection Reagent (Qiagen) as recommended by the manufacturer with minor modifications.[48] After 5 h the transfection medium was changed with serum-free medium, and then the cells were exposed to treatments.

Western blotting and immunoprecipitation

Cells were lysed in ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (aprotinin, PMSF, and sodium orthovanadate). The protein content was determined using Bradford dye reagent (Bio-Rad). Equal amounts of total protein were resolved on SDS–polyacrilamide gels and transferred onto a nitrocellulose membrane as described.[49] Blots were incubated overnight at 4 °C and probed with the specific primary antibodies. Immunoblotting was performed as reported.[50] For immunoprecipitation, 500 μg of total protein lysates were precleared for 1 h with protein A/G-agarose (Santa Cruz Biotechnology), incubated with primary Abs at 4 °C for 18 h in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 0.1 mM Na_3VO_4), and then the antigen-Ab complexes were precipitated with protein A/G agarose for 2 h in HNTG buffer. The immunoprecipitated proteins were washed 3 times with HNTG buffer, separated on SDS-PAGE, and processed by western blotting. The following antibodies were used: AdipoR1, ERα, c-Src, APPL1, EGFR, and GAPDH from Santa Cruz Biotechnology; pERαSer118, pERαSer167, total MAPK and phosphorylated p42/44 MAPK(Thr^{202}/Tyr^{204}), IGF-IR, and pIGF-IR^{Tyr1131} from Cell Signaling Technology.

Kinase activity of c-Src

To assay for c-Src kinase activity MCF-7 cells were grown in PRF-SFM for 24 h and stimulated with 1 or 5 μg/ml adiponectin for 15 min. Cells were then lysed with RIPA lysis buffer (500 mM TRIS-HCl, 150 mM NaCl, 1% Triton X-100) containing 10 mM PMSF, 1.5 mg/ml aprotinin, and 2 mg/ml leupeptin and immunoprecipitated. A Protein G–agarose and an anti-APPL1 antibody complex were prepared to immunopurify the lysates.

A measure of 1 μg of rabbit polyclonal anti-APPL1 antibody and 30 μl of protein G-agarose (Santa Cruz, Biotechnology) were incubated at 4 °C for 1 h in 500 μl of PBS with a tube rotator. The complexes were microfuged and washed with 1 ml of lysis buffer for 3 times. At the end, 1000 μg of each cell lysates were added to the Protein G-agarose/anti-APPL1 antibodies and incubated at 4 °C for 2 h rotating. The proteins/complexes were centrifuged and washed 3 times with the kinase buffer (200 mM PIPES, 100 mM MnCl2). c-Src kinase activity was assayed by a standard in vitro kinase assay using acidified enolase as substrate. The incubation was performed in a total volume of 50 μl composed of the immunoprecipitates and the kinase buffer containing 5 mM ATP, 1 μC of [γ^{32}P]ATP, and 2.5 μg of acid denatured rabbit muscle enolase (Sigma) as exogenous substrate. Samples

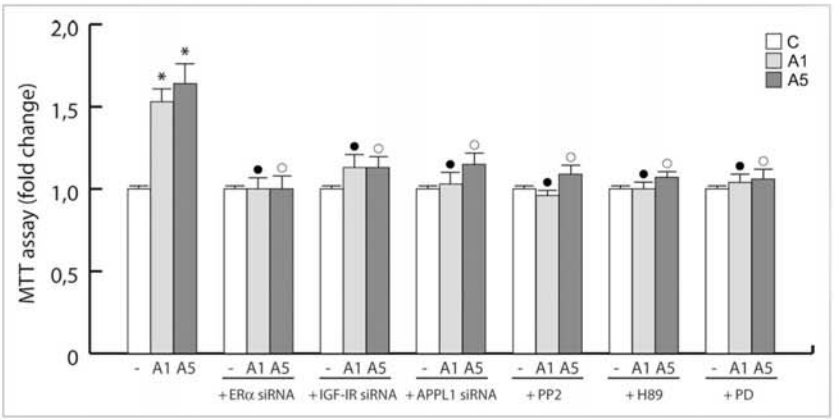

Figure 7. Involvement of the protein complex in the adiponectin-induced MCF-7 cell growth. MTT growth assays in MCF-7 cells untreated or treated for 72 h with A1 and A5 in the absence or presence of ERα, IGF-IR, or APPL1 siRNA, or in combination with PP2, H89, or PD98059 inhibitor of c-Src, PKA, and MAPK, respectively. Cell proliferation is expressed as fold change ± SD relative to vehicle treated cells, and is representative of 3 different experiments each performed in triplicate. *$P < 0.05$ compared with control (–); •$P < 0.05$ compared with A1; ○$P < 0.05$ compared with A5.

were incubated at 30 °C for 10 min then reduced with an equal volume of 2× SDS Laemmli sample buffer (Sigma) and aliquots of them (40 μl) were submitted to SDS-PAGE (acrylamide 11%). The dried gel was exposed to X-omat film (Kodak) for 12 h. The gels were stained with Coomassie blue to ensure that an equal amount of enolase was present in all samples.

Plasmids

XETL plasmid, which carries firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter, was provided by Dr Picard, University of Geneva. S104/106/118A-ER, S118A-ER, and S167A-ER plasmids were mutated in serine residues 104, 106, 118, or 167 to Ala, respectively (a gift from Dr DA Lannigan, University of Virginia); HE241G ERα plasmid mutant that lacks a nuclear translocation signal (NLS) (Δ250–303) (kindly provided by Dr P Chambon, CNRS-INSERM, University of Louis Pasteur). mERα plasmid containing the AF-2/ligand binding domain and a signal that targets this portion of the receptor to the plasma membrane (generously provided by Dr ER Levin, University of California).

Transfections and luciferase assays

MCF-7 cells were transfected using the FuGENE 6 reagent as recommended by the manufacturer with the mixture containing 0.5 μg of reporter plasmid XETL. A set of experiments was performed cotransfecting XETL HeLa cells with XETL and HEGO. Another set of experiments was performed by using 0.5 μg/well pSG5/HE15 (AF-1), pSG5/HE19 (AF-2), HE241G, S104/106/118A-ER, S118A-ER, or S167A-ER plasmids. Six hours after transfection, treatments were added, and cells were incubated for 48 h. A concentration 10 μM, of ICI 182,780 (Tocris Bioscience), Compound C (Enzo Life Sciences), H89 and PP2 (Sigma), PD98059 (Calbiochem), was used. TK *Renilla* luciferase plasmid (25 ng/well) serves to normalize the efficiency of the transfection. Firefly and *Renilla* luciferase activities were measured using a Dual Luciferase kit. The firefly luciferase data for each sample were normalized on the basis of transfection efficiency measured by *Renilla* luciferase activity as reported above.[51]

Immunocytochemical staining

Paraformaldehyde-fixed MCF-7 cells were used for immunocytochemical staining. Endogenous peroxidase activity was inhibited by hydrogen peroxide, and nonspecific sites were blocked by normal horse serum. ERα immunostaining was then performed using as primary antibody a mouse monoclonal antiserum, whereas a biotinylated horse-anti-mouse IgG was utilized as secondary antibody. Avidin–biotin–horseradish peroxidase complex (ABC complex/horseradish peroxidase) was applied, and the chromogen 3,3′-diaminobenzidine tetrachloride dihydrate was used as detection system. TBS-T (0.05 M TRIS-HCl plus 0.15 M NaCl, pH 7.6 containing 0.05% Triton X-100) served as washing buffer. The primary antibody was replaced by normal mouse serum at the same concentration in control experiments on MCF-7 cultured cells.

Reverse transcription-PCR assay

The gene expression of ERα, cathepsin D, pS2, and 36B4 was evaluated by reverse transcription PCR (RT-PCR) method as described.[52] Primer sequences include: estrogen receptor α (*ERα*), forward 5′-TGATTGGTCT CGTCTGGCG-3′ and reverse 5′-CATGCCCTCT ACACATTTTC CC-3′; Cathepsin D (*CatD*), forward 5′-AACAACAGGG TGGGCTTC-3′and reverse 5′-TTTGAGTAGT CAAAGTCAGA GC-3′; Trefoil factor 1/pS2 (*pS2*), forward 5′-TTCTATCCTA ATACCATCGA CG-3′ and reverse 5′-TTTGAGTAGT CAAAGTCAGA GC-3′; 36B4, forward 5′-CTCAACATCT CCCCCTTCTC-3′ and reverse 5′- CAAATCCCAT ATCCTCGT -3′. Equal amounts of PCR product were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

Statistical analysis

Each datum point represents the mean ± SD of at least 3 independent experiments. Data were analyzed by Student *t* test using the GraphPad Prism 4 software program. $P < 0.05$ was considered as statistically significant.

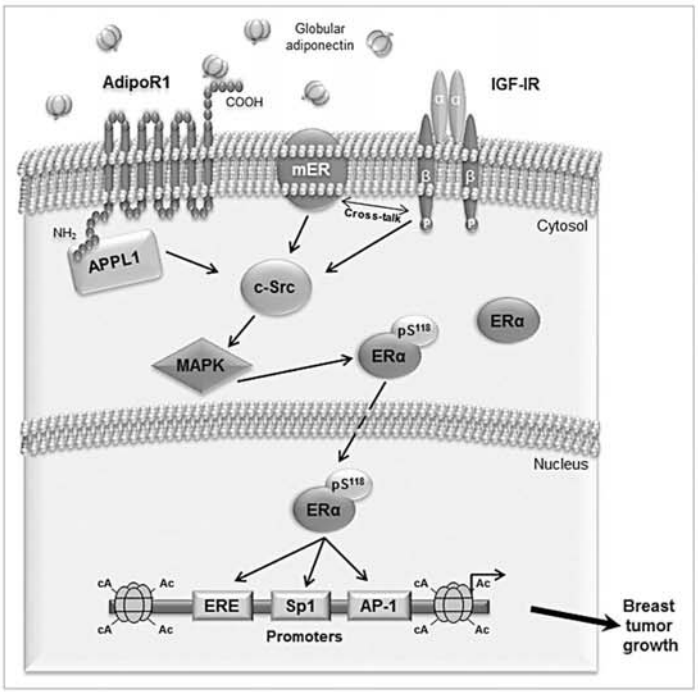

Figure 8. Proposed model of adiponectin-induced proliferation in ERα-positive breast cancer cells. Globular adiponectin binds to AdipoR1, which interacts with the adaptor protein APPL1, through the intracellular N terminus. Adiponectin induces physical interaction between AdipoR1/APPL1, membrane ERα, IGF-IR, and c-Src, leading to MAPK phosphorylation. This contributes to ERα activation at genomic level through the phosphorylation at Ser118. Consequently, adiponectin, increasing ERα transactivation, positively affects MCF-7 cell growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC) [MFAG grant number 6180 and IG grant number 11595]. Progetti di Ricerca di Interesse Nazionale (PRIN)-Ministero Istruzione Università e Ricerca (MIUR) [grant number 20085Y7XT5]. European Commission/FSE/Regione Calabria to Emilia Ricchio.

The authors thank Dr Pasquale Cicirelli for his technical assistance.

References

1. MacDougald OA, Burant CF. The rapidly expanding family of adipokines. Cell Metab 2007; 6:159-61; PMID:17767903; http://dx.doi.org/10.1016/j.cmet.2007.08.010
2. Andò S, Catalano S. The multifactorial role of leptin in driving the breast cancer microenvironment. Nat Rev Endocrinol 2012; 8:263-75; PMID:22083089; http://dx.doi.org/10.1038/nrendo.2011.184
3. Brochu-Gaudreau K, Rehfeldt C, Blouin R, Bordignon V, Murphy BD, Palin MF. Adiponectin action from head to toe. Endocrine 2010; 37:11-32; PMID:20963555; http://dx.doi.org/10.1007/s12020-009-9278-8
4. Matsubara M, Maruoka S, Katayose S. Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women. Eur J Endocrinol 2002; 147:173-80; PMID:12153737; http://dx.doi.org/10.1530/eje.0.1470173
5. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, Kahn SE. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 2003; 46:459-69; PMID:12687327
6. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 2001; 86:1930-5; PMID:11344187; http://dx.doi.org/10.1210/jc.86.5.1930
7. Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, et al. Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem 2002; 277:25863-6; PMID:12032136; http://dx.doi.org/10.1074/jbc.C200251200
8. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat Med 2002; 8:731-7; PMID:12068289; http://dx.doi.org/10.1038/nm724
9. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 2006; 6:772-83; PMID:16998510; http://dx.doi.org/10.1038/nri1937
10. Dalamaga M, Diakopoulos KN, Mantzoros CS. The role of adiponectin in cancer: a review of current evidence. Endocr Rev 2012; 33:547-94; PMID:22547160; http://dx.doi.org/10.1210/er.2011-1015
11. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc Natl Acad Sci U S A 2001; 98:2005-10; PMID:11172066; http://dx.doi.org/10.1073/pnas.98.4.2005
12. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 2003; 423:762-9; PMID:12802337; http://dx.doi.org/10.1038/nature01705
13. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. Endocr Rev 2005; 26:439-51; PMID:15897298; http://dx.doi.org/10.1210/er.2005-0005
14. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, et al. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol 2006; 8:516-23; PMID:16622416; http://dx.doi.org/10.1038/ncb1404
15. Lee M-H, Klein RL, El-Shewy HM, Luttrell DK, Luttrell LM. The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/Ras-dependent pathway and stimulate cell growth. Biochemistry 2008; 47:11682-92; PMID:18842004; http://dx.doi.org/10.1021/bi801451f
16. Kelesidis I, Kelesidis T, Mantzoros CS. Adiponectin and cancer: a systematic review. Br J Cancer 2006; 94:1221-5; PMID:16570048; http://dx.doi.org/10.1038/sj.bjc.6603051
17. Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, Noguchi S. Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 2003; 9:5699-704; PMID:14654554
18. Mantzoros C, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, Papadiamantis Y, Markopoulos C, Spanos E, Chrousos G, et al. Adiponectin and breast cancer risk. J Clin Endocrinol Metab 2004; 89:1102-7; PMID:15001594; http://dx.doi.org/10.1210/jc.2003-031804
19. Körner A, Pazaitou-Panayiotou K, Kelesidis T, Kelesidis I, Williams CJ, Kaprara A, Bullen J, Neuwirth A, Tseleni S, Mitsiades N, et al. Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. J Clin Endocrinol Metab 2007; 92:1041-8; PMID:17192291; http://dx.doi.org/10.1210/jc.2006-1858
20. Kang JH, Lee YY, Yu BY, Yang BS, Cho KH, Yoon DK, Roh YK. Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. Arch Pharm Res 2005; 28:1263-9; PMID:16350853; http://dx.doi.org/10.1007/BF02978210
21. Dos Santos E, Benaitreau D, Dieudonne MN, Leneveu MC, Serazin V, Giudicelli Y, Pecquery R. Adiponectin mediates an antiproliferative response in human MDA-MB 231 breast cancer cells. Oncol Rep 2008; 20:971-7; PMID:18813842
22. Grossmann ME, Nkhata KJ, Mizuno NK, Ray A, Cleary MP. Effects of adiponectin on breast cancer cell growth and signaling. Br J Cancer 2008; 98:370-9; PMID:18182989; http://dx.doi.org/10.1038/sj.bjc.6604166
23. Dieudonne MN, Bussiere M, Dos Santos E, Leneveu MC, Giudicelli Y, Pecquery R. Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. Biochem Biophys Res Commun 2006; 345:271-9; PMID:16678125; http://dx.doi.org/10.1016/j.bbrc.2006.04.076
24. Jardé T, Caldefie-Chézet F, Goncalves-Mendes N, Mishellany F, Buechler C, Penault-Llorca F, Vasson MP. Involvement of adiponectin and leptin in breast cancer: clinical and in vitro studies. Endocr Relat Cancer 2009; 16:1197-210; PMID:19661131; http://dx.doi.org/10.1677/ERC-09-0043
25. Treeck O, Lattrich C, Juhasz-Boess I, Buchholz S, Pfeiler G, Ortmann O. Adiponectin differentially affects gene expression in human mammary epithelial and breast cancer cells. Br J Cancer 2008; 99:1246-50; PMID:18827813; http://dx.doi.org/10.1038/sj.bjc.6604692
26. Pfeiler GH, Buechler C, Neumeier M, Schäffler A, Schmitz G, Ortmann O, Treeck O. Adiponectin effects on human breast cancer cells are dependent on 17-beta estradiol. Oncol Rep 2008; 19:787-93; PMID:18288417
27. Landskroner-Eiger S, Qian B, Muise ES, Nawrocki AR, Berger JP, Fine EJ, Koba W, Deng Y, Pollard JW, Scherer PE. Proangiogenic contribution of adiponectin toward mammary tumor growth in vivo. Clin Cancer Res 2009; 15:3265-76; PMID:19447867; http://dx.doi.org/10.1158/1078-0432.CCR-08-2649
28. Sflomos G, Brisken C. A new Achilles Heel in breast cancer? Oncotarget 2013; 4:1126-7; PMID:23907591
29. Mauro L, Surmacz E. IGF-I receptor, cell-cell adhesion, tumour development and progression. J Mol Histol 2004; 35:247-53; PMID:15339044; http://dx.doi.org/10.1023/B:HIJO.0000032356.98363.a2
30. Simoncini T, Genazzani AR. Non-genomic actions of sex steroid hormones. Eur J Endocrinol 2003; 148:281-92; PMID:12611608; http://dx.doi.org/10.1530/eje.0.1480281
31. Schäffler A, Schölmerich J, Buechler C. Mechanisms of disease: adipokines and breast cancer - endocrine and paracrine mechanisms that connect adiposity and breast cancer. Nat Clin Pract Endocrinol Metab 2007; 3:345-54; PMID:17377617; http://dx.doi.org/10.1038/ncpendmet0456
32. Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose tissue on cancer development and progression. Endocr Rev 2011; 32:550-70; PMID:21642230; http://dx.doi.org/10.1210/er.2010-0030
33. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 2004; 89:2548-56; PMID:15181022; http://dx.doi.org/10.1210/jc.2004-0395
34. Trujillo ME, Scherer PE. Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 2005; 257:167-75; PMID:15656875; http://dx.doi.org/10.1111/j.1365-2796.2004.01426.x
35. Jardé T, Perrier S, Vasson MP, Caldefie-Chézet F. Molecular mechanisms of leptin and adiponectin in breast cancer. Eur J Cancer 2011; 47:33-43; PMID:20889333; http://dx.doi.org/10.1016/j.ejca.2010.09.005
36. Wang Y, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, Wu D, Cooper GJ, Xu A. Adiponectin modulates the glycogen synthase kinase-3β/β-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Res 2006; 66:11462-70; PMID:17145894; http://dx.doi.org/10.1158/0008-5472.CAN-06-1969
37. Chen X, Wang Y. Adiponectin and breast cancer. Med Oncol 2011; 28:1288-95; PMID:20625941; http://dx.doi.org/10.1007/s12032-010-9617-x

38. Lanzino M, Morelli C, Garofalo C, Panno ML, Mauro L, Andò S, Sisci D. Interaction between estrogen receptor alpha and insulin/IGF signaling in breast cancer. Curr Cancer Drug Targets 2008; 8:597-610; PMID:18991569; http://dx.doi.org/10.2174/156800908786241104
39. Song RX, Chen Y, Zhang Z, Bao Y, Yue W, Wang JP, Fan P, Santen RJ. Estrogen utilization of IGF-1-R and EGF-R to signal in breast cancer cells. J Steroid Biochem Mol Biol 2010; 118:219-30; PMID:19815064; http://dx.doi.org/10.1016/j.jsbmb.2009.09.018
40. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001; 22:153-83; PMID:11294822; http://dx.doi.org/10.1210/er.22.2.153
41. Deepa SS, Dong LQ. APPL1: role in adiponectin signaling and beyond. Am J Physiol Endocrinol Metab 2009; 296:E22-36; PMID:18854421; http://dx.doi.org/10.1152/ajpendo.90731.2008
42. Murphy LC, Weitsman GE, Skliris GP, Teh EM, Li L, Peng B, Davie JR, Ung K, Niu YL, Troup S, et al. Potential role of estrogen receptor alpha (ERalpha) phosphorylated at Serine118 in human breast cancer in vivo. J Steroid Biochem Mol Biol 2006; 102:139-46; PMID:17092701; http://dx.doi.org/10.1016/j.jsbmb.2006.09.021
43. Luttrell LM. 'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors. J Mol Endocrinol 2003; 30:117-26; PMID:12683936; http://dx.doi.org/10.1677/jme.0.0300117
44. De Amicis F, Russo A, Avena P, Santoro M, Vivacqua A, Bonofiglio D, Mauro L, Aquila S, Tramontano D, Fuqua SA, et al. In vitro mechanism for downregulation of ER-α expression by epigallocatechin gallate in ER+/PR+ human breast cancer cells. Mol Nutr Food Res 2013; 57:840-53; PMID:23322423; http://dx.doi.org/10.1002/mnfr.201200560
45. Rovito D, Giordano C, Vizza D, Plastina P, Barone I, Casaburi I, Lanzino M, De Amicis F, Sisci D, Mauro L, et al. Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. J Cell Physiol 2013; 228:1314-22; PMID:23168911; http://dx.doi.org/10.1002/jcp.24288
46. Panno ML, Giordano F, Rizza P, Pellegrino M, Zito D, Giordano C, Mauro L, Catalano S, Aquila S, Sisci D, et al. Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination. Breast Cancer Res Treat 2012; 136:443-55; PMID:23053665; http://dx.doi.org/10.1007/s10549-012-2282-3
47. Mauro L, Catalano S, Bossi G, Pellegrino M, Barone I, Morales S, Giordano C, Bartella V, Casaburi I, Andò S. Evidences that leptin up-regulates E-cadherin expression in breast cancer: effects on tumor growth and progression. Cancer Res 2007; 67:3412-21; PMID:17409452; http://dx.doi.org/10.1158/0008-5472.CAN-06-2890
48. Guido C, Panza S, Santoro M, Avena P, Panno ML, Perrotta I, Giordano F, Casaburi I, Catalano S, De Amicis F, et al. Estrogen receptor beta (ERβ) produces autophagy and necroptosis in human seminoma cell line through the binding of the Sp1 on the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) promoter gene. Cell Cycle 2012; 11:2911-21; PMID:22810004; http://dx.doi.org/10.4161/cc.21336
49. Mauro L, Pellegrino M, Lappano R, Vivacqua A, Giordano F, Palma MG, Andò S, Maggiolini M. E-cadherin mediates the aggregation of breast cancer cells induced by tamoxifen and epidermal growth factor. Breast Cancer Res Treat 2010; 121:79-89; PMID:19593637; http://dx.doi.org/10.1007/s10549-009-0456-4
50. Guido C, Whitaker-Menezes D, Lin Z, Pestell RG, Howell A, Zimmers TA, Casimiro MC, Aquila S, Ando' S, Martinez-Outschoorn UE, et al. Mitochondrial fission induces glycolytic reprogramming in cancer-associated myofibroblasts, driving stromal lactate production, and early tumor growth. Oncotarget 2012; 3:798-810; PMID:22878233
51. De Amicis F, Zupo S, Panno ML, Malivindi R, Giordano F, Barone I, Mauro L, Fuqua SA, Andò S. Progesterone receptor B recruits a repressor complex to a half-PRE site of the estrogen receptor alpha gene promoter. Mol Endocrinol 2009; 23:454-65; PMID:19147702; http://dx.doi.org/10.1210/me.2008-0267
52. Casaburi I, Avena P, Lanzino M, Sisci D, Giordano F, Maris P, Catalano S, Morelli C, Andò S. Chenodeoxycholic acid through a TGR5-dependent CREB signaling activation enhances cyclin D1 expression and promotes human endometrial cancer cell proliferation. Cell Cycle 2012; 11:2699-710; PMID:22751440; http://dx.doi.org/10.4161/cc.21029

Anticancer Activity of a Hydrogel Containing Folic Acid Towards MCF-7 and MDA-MB-231 Cells

SONIA TROMBINO, TERESA FERRARELLI, MICHELE PELLEGRINO, EMILIA RICCHIO, LOREDANA MAURO, SEBASTIANO ANDÒ, NEVIO PICCI and ROBERTA CASSANO

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Cosenza, Italy

Abstract. *Aim: The aim of the present study was to prepare a hydrogel, based on ellagic acid and glycine, embedded with folic acid, as a subcutaneous implant for the treatment of breast cancer. The function of folic acid is to selectively and actively target tumor cells which are well-known to overexpress folic acid receptors on their surface. Materials and Methods: A pro-drug based on L-glycine and ellagic acid was functionalized with a polymerizable group and loaded with folic acid to make it more natural, non-toxic, compatible and specific for the site of action. Cytotoxicity against MCF-7 cells was also evaluated. Release studies of folic acid were conducted on aliquots of hydrogel at different pH (6.2 and 7.4) and time-points (1, 6, 12 and 24 h) using a shaking water bath at 37°C (body temperature). Results: Our results show that folic acid release by the hydrogel is characterized by a slow kinetic release, especially at pH 6.2. Moreover, it was evidenced that the exposure of human breast cancer cells to ellagic acid-based hydrogel containing folic acid significantly reduced cell viability.*

The use of polymeric materials in the pharmaceutical fields has enabled for creation of various drug delivery systems based on a wide range of biocompatible polymers (1-4) with hydrophilic structures and different physicochemical properties (5, 6). In particular among all of these polymers, hydrogels allow for preparation of modified drug delivery systems, both in terms of time and in terms of targeting (7, 8). Hydrogels are crosslinked polymeric structures that can absorb great quantities of water or biological fluids. Due to the nature of their hydrophilic chains, hydrogels are thermodynamically compatible with water. This feature allows them to swell in aqueous media. Hydrogels have many applications in the biomedical and pharmaceutical fields because, as a result of their properties, they are very similar to living tissue compared to other synthetic materials. For these reasons they have been used in contact lenses, membranes for biosensors, and as materials for the construction of artificial skin and devices for releasing drugs and proteins (9, 10).

Ellagic acid is a depside, present in dried fruits and berries, including raspberries, strawberries, blackberries, cranberries, pomegranates and walnuts, with different EAHling properties such as anti-oxidant, anti-bacterial, anticancer and anti-viral (11-14). Numerous studies have shown that ellagic acid has anticancer activity towards tumor cells of breast, esophagus, pancreas, skin and prostate (15-17). The mechanism of action of ellagic acid on tumor cells consists in the inhibition and in the blocking of their replication and in the induction of their apoptosis (18).

The purpose of this work was to design, prepare and characterize a potentially useful prodrug for breast cancer treatment. In particular, a L-glycine and ellagic acid-based derivative was functionalized with a polymerizable group, polymerized and loaded with folic acid (FA) (Figure 1).

The function of FA in this case is to selectively and actively target the attachment of the implant towards tumor cells, which are known to overexpress FA receptors on their surface (19-21). To achieve this goal, L-glycine and ellagic acid, were functionalized with a polymerizable group and loaded with FA to make it more natural, non-toxic, compatible and specific for the site of action. The obtained compounds were characterized by Fourier Transform Infrared (FT-IR) and Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectrometries. The ability of the obtained hydrogel to protect against lipid peroxidation induced by *tert*-BOOH, was examined in rat liver microsomal membranes. Cytotoxicity towards breast cancer cells was also evaluated. The data showed a significant decrease in viability of cells exposed to hydrogel ellagic acid-based containing folic acid.

Correspondence to: Roberta Cassano, Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Edificio Polifunzionale, Via Pietro Bucci, 87036 Arcavacata di Rende, Cosenza, Italy. Tel/Fax: +39 984493227, e-mail: roberta.cassano@unical.it

Key Words: Hydrogel, ellagic acid, glycine, folic acid, anti-oxidant, breast cancer, implant.

0250-7005/2013 $2.00+.40

The results of all studies indicated the possibility that the ellagic acid-based hydrogel may find use, as subcutaneous implant, in the treatment of tumors whose conventional therapy provides the medication causing the onset of serious side-effects.

Materials and Methods

Reagents. All solvents of analytical grade were purchased from Carlo Erba Reagents (Milan, Italy): acetone, chloroform, dichloromethane, ethanol, ethyl ether, methanol, *n*-hexane, acetonitrile and triethylamine. L-Glycine (MW=75.07), 4,4'-dimetoxytrityl chloride (DMTrCl), trimethyl chlorosilane (Me_3SiCl), ellagic acid (MW=302.19), dicyclohexyl carbodiimide (DCC), dimethylaminoyiridine (DMAP), acryloyl chloride, ammonium persulfate $(NH4)_2S_2O_8$, *N,N*-dimethylacrylamide (DMAA), FA, *tert*-butyl hydroperoxide (t-BOOH) trichloroacetic acid (TCA) acid, 2-thiobarbituric (TBA), butylated hydroxytoluene (BHT), 4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA).

Cell culture. MCF-7 and MDA-MB-231 cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 containing 5% fetal bovine serum and supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma, Milan, Italy). The cells were cultured in serum-free medium (SFM) for at least 24 h before treatments.

Instruments. Infrared spectra were performed on KBr pellets using an FT-IR Perkin-Elmer 1720 spectometer (Norwalk, Connecticut, USA), in the range 4000-400 cm^{-1} (16 scans). The ^{1}H-MNR were performed by a Bruker VM30 spectometer (Bruker, Zürich, Switzerland), the chemical shifts are expressed in δ and are related to the solvent. The structures of synthesized compounds was confirmed by (GC-MS) Hewlett Packard 5972 (HAZLET, New Jersey, USA). The UV-VIS spectra were carried out using JASCO-530 UV spectrophotometer (JASCO Europe s.r.l., Milan, Italy). Samples were freeze-dried using a freeze-drying Micro Modulyo Edwards apparatus (Hastings, UK).

Synthesis of N-trytilglycine (1). The reaction was conducted according to the procedure reported in literature (22). In a three-neck flask fitted with a reflux condenser, funnel dripper, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, L-glycine (0.27 g, 13 mmoles) was dissolved in 1.22 ml mixture of chloroform (0.98 ml) and acetonitrile (0.24 ml). The reaction mixture was maintained at 30°C under magnetic stirring. After dissolution, 0.42 ml (3.3 mmol) of trimethyl chlorosylane were added. The reaction was conducted for 2 h at 30°C. Thereafter, 0.97 ml (6.9 mmol) of dry triethylamine and dropwise 4,4'-dimethoxytrytil chloride (1.20 g, 13 mmol), dissolved in chloroform (15.77 ml) were added. The addition of 4,4'-dimethoxytrytil chloride caused a chromatic change from white to red. This mixture was maintained for one hour under magnetic stirring; then 0,71 ml of methanol were added causing chromatic change from red to grey-green. The mixture was left stirring for 2 h. Finally the colour was pale yellow. The reaction was monitored through (TLC) on aluminum oxide (eluent mixture: chloroform). The solvents were then evaporated under reduced pressure and the obtained solid was washed with diethyl ether and then with an aqueous solution of citric acid 5% w/v. The organic phase was treated with a solution of NaOH 1 N (7.18 ml) and water (3.59 ml). The phase was treated with cold diethyl ether (17.18 ml) and neutralized with glacial acetic acid and the obtained precipitate was extracted with diethyl ether, while the organic phase was washed with distilled water and dried for 2 h with magnesium sulphate. The solution was filtered and dried under reduced pressure. The obtained product (**1**), orange in colour, was characterized through FT-IR.

Esterification of N-trytilglycine with ellagic acid (2). The reaction was conducted in agreement with the procedure reported in literature (23). In a two-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, 0.50 g (1.32 mmol) of (**1**) and 0.39 g (1.32 mmol) of ellagic acid were dissolved in 2.64 ml of dry dichloromethane. After dissolution, DCC (0.35 g, 1.7 mmol) and DMAP (0.08 g, 0.65 mmol) were added. The reaction mixture was maintained at room temperature under magnetic stirring for 12 hours. The reaction was monitored through TLC on silica gel (eluent mixture chloroform/n-exane 5:5). Finally, the product was filtered to be purified from dicyclohexyl urea formed during the reaction. After that it was purified through a chromatographic column on silica gel (eluent mixture dichloromethane/petroleum ether 4:6). The obtained product (**2**) was characterized through FT-IR.

Detritylation reaction (3). The reaction was conducted in agreement with the procedure reported in literature (24). In a three-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, 0.15 g of (**2**) (0.22 mmol) were added to a solution of trifluoroacetic acid in dichloromethane 60% giving a red-colored solution. After that, 0.45 ml of methanol (0.59 mmol) were added and the mixture was stirred for one hour at room temperature until the observation of a chromatic shift from red to yellow-orange. The solvent was evaporated at reduced pressure and the obtained salt was dried. This latter was subsequently washed with 2.26 ml of a 1 N HCl solution in MeOH. The solvent was evaporised and the product was dried and washed with diethyl ether in order to eliminate the trytil moieties. The obtained product (3) was dried under reduced pressure and characterized through FT-IR and ^{1}H-NMR.

Acrylation of 3. In a three-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, product **3** (0.30 g, 0.83 mmol) was dissolved in 6 ml of dichloromethane. After that, 0.08 ml (0.99 mmol) of acryloil chloride and 0.14 ml (0.1 mmol) of triethylamine were added and the solution appeared yellow coloured. The reaction was maintained for 12 hours at room temperature and was monitored through TLC on silica gel (eluent mixture chloroform/methanol 9:1). Finally, the solvent was evaporated under reduced pressure and the obtained product (**4**) was characterized by FT-IR e ^{1}H-NMR.

Hydrogel preparation. In a two-neck flask fitted with a reflux condenser, magnetic stirring thoroughly flamed and maintained under nitrogen bubbling, product **4** (0.65 g, 1.57 mmol) was solubilized in an aqueous solution of NH_3/urea. Subsequently, DMAA (0.16 ml, 0.1 mmol) and ammonium persulphate (80.35 g, 0.35 mol) were added. Finally, the reaction was carried out at 60°C

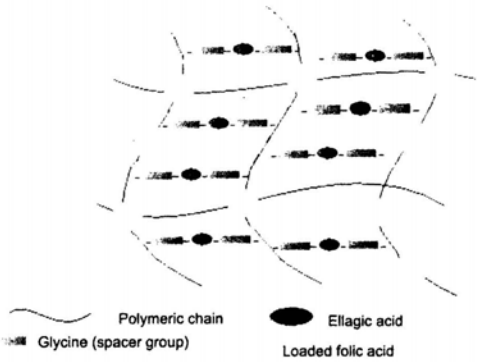

Figure 1. *Ellagic acid and glicine-based hydrogel containing folic acid.*

until the formation of the hydrogel. The obtained hydrogel was washed with diethyl ether in a porous filter, dried under vacuum and characterized through FT-IR (9).

Antioxidant activity evaluation. The ability of the obtained hydrogel to protect against lipid peroxidation induced by *tert*-BOOH, was examined in rat liver microsomal membranes during 120 min of incubation. Aliquots of both ellagic acid and ellagic acid-based hydrogel (EAH) were added to the microsomal suspension (9, 25-27). Then the suspensions were incubated at 37°C in a shaking bath under air in the dark. After incubation, thiobarbituric acid-malondialdehyde complex (TBA-MDA) formation was monitored by the use of UV-VIS spectrophotometry at 535 nm (25). The experiment was repeated in triplicate (n=3).

Swelling studies. The swelling characteristics of the hydrogel were determined in order to check for its hydrophilic affinity (9). Typically, aliquots (40-50 mg) of hydrogel dried to constant weight were placed on a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighed, and left to swell by immersing the filter plus support in a beaker containing the swelling media, namely phosphate buffers at pH 6.2 to mimic the conditions typical of tumor pathology, and pH 7.4 to simulate the physiological environment. At a predetermined time, the excess of water was removed by percolation at atmospheric pressure. Then the filter was placed in a centrifuge test tube, fixed with the help of a bored silicone stopper, then centrifuged at 1372 rcf for 15 min and weighed. This operation was repeated at the different times (1, 6, 12 and 24 h). The filter weight was determined after centrifugation with water alone. The weights recorded at the different times were averaged and used to give the water content (α, %) by the following equation:

$$\alpha\% = \frac{(Ws-Wd)}{Wd} \times 100$$

where Ws and Wd are weights of swollen and dried hydrogel, respectively.

Incorporation of FA into preformed hydrogel. The folic acid incorporation into microspheres was performed (9) as follows: 500 mg of preformed empty hydrogel (prepared as described above) were wetted with 6 ml in a concentrated solution of folic acid (15 mg/ml). After three days, under slow stirring at 37°C, the microspheres were filtered and dried at reduced pressure in the presence of P_2O_5 to a constant weight. The loading efficiency (LE, %) of all samples were determined by UV-Vis spectrophotometric analysis of filtered solvent according to the following equation:

$$LE(\%) = Ci - C0/Ci \times 100$$

where C_i was the concentration of FA in solution before the loading study, C_0 the concentration of FA in solution after the loading study. LE was measured spectrophotometrically (λ=365 nm, ε=7595 $mol^{-1}dm^3cm^{-1}$).

Drug release studies. Dried hydrogel (10 mg) was dispersed in 6 ml of swelling media (phosphate buffers at pH 6.2 and pH 7.4 (9). The test tubes were maintained at 37°C in an horizontal shaking bath and shaked at a rate of 100 rpm. At predetermined intervals, the samples were centrifuged, 5 ml of supernatant were removed and the medium was replaced with fresh solution to maintain the same total volume throughout the study. The concentration of FA was determined spectrophotometrically at 365 nm. The experiment was repeated in triplicate (n=3). The data were calculated in terms of the drug release percentage.

(MTT) assay. The cytotoxic effects of EAH and of EAH containing folic acid on human breast cancer cells were evaluated by MTT dye test (cell viability) (27, 28). The cells were seeded at a density of 1×10^4 cells in 96-well tissue culture plates for 24 h at 37°C and 5% CO_2 to allow the adhesion of the cells. After 24 hours of incubation, the culture medium was replaced with SFM and EAH or EAH+FA were added at 50 μM. The toxicity experiments were carried out at 24 and 48 h of incubation. Untreated cells were used as control.

At the end of the incubation time, 50 μl of MTT tetrazolium salt (5 mg/ml dissolved in SFM) were added to each well. The plates were incubated for an additional four hours and then the medium was discarded. A volume of 150 μl of DMSO was added to each well, and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (Multiskan EX, Thermo Scientific, USA) at a test wavelength of 570 nm with a reference wavelength of 690 nm. The sample was tested in triplicate and the percentage of viable cells, directly proportional to the amount of formazan crystals formed, was calculated using the following equation: % Cell viability=AbsT/AbsU ×100, where AbsT is the absorbance of treated cells and AbsU is the absorbance of untreated cells.

Statistical analysis. Data are expressed as the mean±S.D. of at least three independent experiments. Statistical analysis was performed using Student's *t*-test. *p*-Values ≤0.05 were considered statistically significant.

Results and Discussion

In recent years, the interest in more greatly biocompatible materials, useful for the controlled and site-specific release of active molecules, has been increasing. In this context the aim of this work was the preparation of a hydrogel, made of ellagic acid and L-glycine, to be used as a subcutaneous implant.

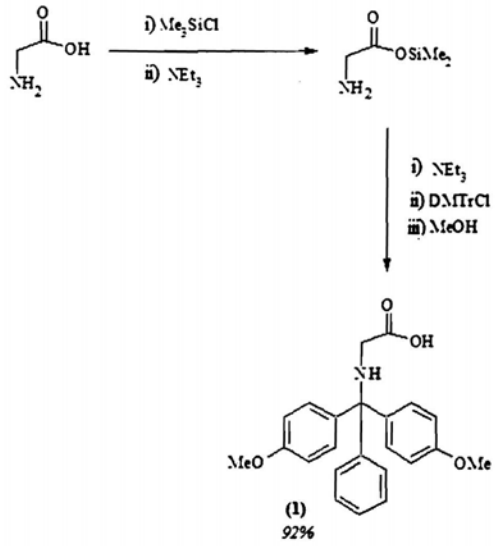

Figure 2. *Synthetic route to N-trytil glycine.*

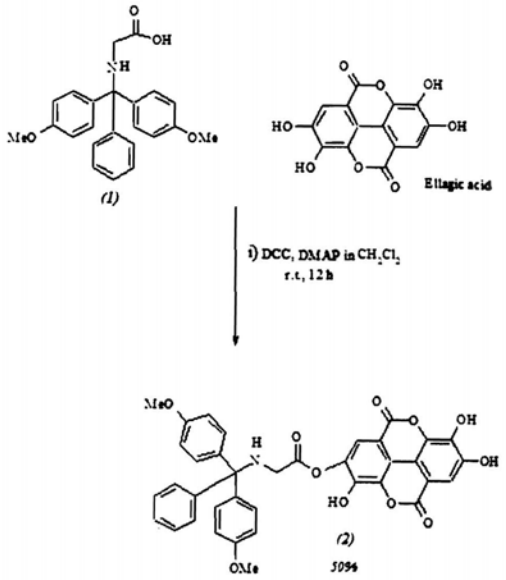

Figure 3. *Esterification reaction.*

L-Glycine is an amino acid that, having two sites of functionalization, can act as a spacer group between an active substance, such as ellagic acid, and a polymerizable group such as acrylic acid. In fact, thanks to the presence of an acrylic moiety, it is possible to perform co-polymerization reactions to obtain a hydrogel potentially employable as subcutaneous implant in the treatment of breast cancer.

The first step of the whole process was the protection of the aminic group of glycine conducted in the presence of trimethyl chlorosilane, a temporary protecting group for the carboxylic function. For this purpose, the reaction was carried out using triethylamine and methanol to enhance the binding of trimethyl chlorosilane and its elimination after protection of the aminic group. DMTrCl was chosen as protecting group of the aminic moiety since it is very effective but easily removable one. Both the protections, the temporary one for –COOH and the stable one for $-NH_2$, were used in stoichiometric amounts. The most likely mechanism of action involves the binding of the carboxyl group of glycine with Me^3SiCl, releasing a mole of hydrochloric acid. After that DMTrCl was added and it was attacked on its electrophilic carbon by the nucleophilic $-NH_2$ releasing another mole of hydrochloric acid. The addition of methanol resulted in the liberation of Me_3SiCl from the carboxyl group of glycine (Figure 2). The product of this first step was characterized by the most common spectroscopic techniques: FT-IR (KBr) ν (cm^{-1}): 3066 e 3035 (aromatics -CH), 1725 (-COOH), 1654 (-CONH). M/Z: 320 (63 %), 77 (38 %). ^{1}H-NMR ($CDCl_3$) δ (ppm): 9.80 (s, 1H), 6.90-7.94 (m, 13H), 4.04 (s, 2H), 3.85 (s, 6H) 3.61 (sb, 1H). Yield: 92%.

The second step of synthesis was the esterification of the carboxyl group of glycine with one of the hydroxyl groups of ellagic acid. For this condensation, a coupling agent such as DCC that binds the -OH group of -COOH makes it become more electrophilic towards nucleophilic attack of hydroxyl groups of ellagic acid. A base, DMAP, was used as buffer system and nucleophilic catalyst to promote the deprotonation of the hydroxyl groups of ellagic acid that become more nucleopilic. The esterification involved the formation of dicyclohexyl urea (DCU) as a leaving group that can be removed by filtration (Figure 3). The obtained product (**2**) was dried under vacuum, purified through a chromatographic column and characterized through FT-IR and ^{1}H-NMR: FT-IR (KBr) ν (cm^{-1}): 3068 e 3034 (aromatics -CH), 1769 (-C=O ester), 1718 (-C=O ester), 1653 (-CONH). ^{1}H-NMR ($CDCl_3$) δ (ppm): 6.80-7.88 (m, 15H), 3.91 (s, 2H), 3.82 (s, 6H). Yield: 50%.

The third step involved the removal of trityl group linked to the amino group of glycine in order to make it free for the next step of acrylation. This protecting group was removed by using a solution of trifluoroacetic acid 60% v/v in dichloromethane. This reaction gives the corresponding

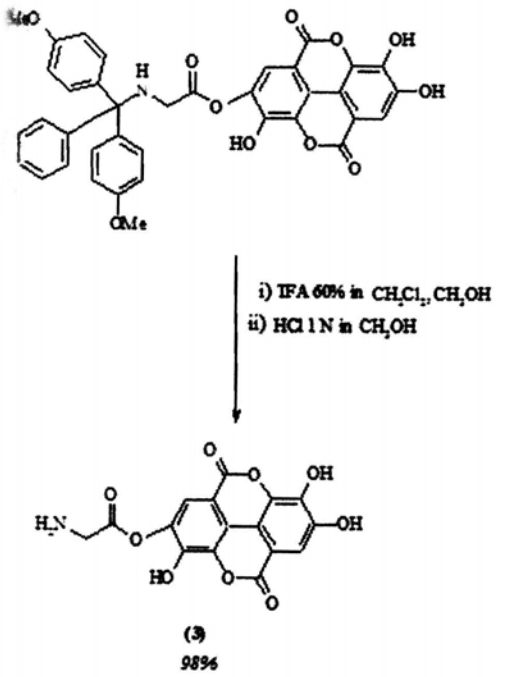

Figure 4. *Deprotection*.

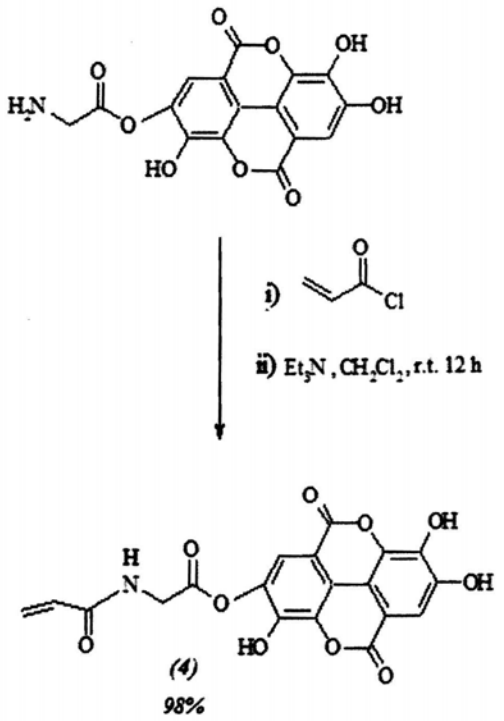

Figure 5. *Acrylation*.

chlorhydrate amine (-NH_2 ×HCl); to obtain the free aminic group, it was necessary to add a solution of 1 N hydrochloric acid in methanol to the dry salt. In order to remove all the free protecting groups the product (**3**) was washed with diethyl ether dried under vacuum and characterized through FT-IR and ^{1}H-NMR (Figure 4). FT-IR shows the disappearance of the amidic band at 1653 cm^{-1} and the preservation of characteristic stretching of ester bonds: ^{1}H-NMR (C_2D_6SO) δ (ppm): 7.25 (s, 1H), 7.10 (s, 1H), 4.04 (s, 2H). Yield: 98%.

The hydroxyl groups of ellagic acid linked to glycine were esterified using acryloyl chloride. The mechanism of this reaction consisted in the nucleophilic attack of the amino group of L-glycine towards the electrophilic carbonyl of acryloil chloride by liberation of HCl (Figure 5). The insertion of an acrylic moiety was important for the step of co-polymerization. The obtained product (4) was characterized through FT-IR and ^{1}H-NMR: FT-IR (KBr) ν (cm^{-1}): 3067, 3034 (aromatics -CH), 1767 (-C=O ester), 1722 (-C=O ester), 1661 (-CONH), 995, 914 (vinyl group). 1H-NMR (C_2D_6SO) δ (ppm): 7.30 (s, 1H), 7.05 (s, 1H), 6.43 (dd, 1H), 6.11 (dd, 1H), 5.80 (dd, 1H), 4.10 (s, 2H). Yield: 98%.

The product (4) was dissolved in a NH_3/urea aqueous solution then ammonium persulfate and dimethyl acrylamide, a monofunctional co-monomer, were added at a temperature of 60°C. The reaction was carried out until the formation of the hydrogel occurred and in the end the obtained product was washed several times with distilled water, dried under vacuum and characterized through FT-IR. This latter shows the disappearance of the bands attributable to the acrylic double bond.

The ability of the obtained hydrogel to inhibit lipid peroxidation in rat liver microsomal membranes during 120 minutes of incubation was examined and compared to the antioxidant activity of free ellagic acid. The results revealed (Figure 6) that the ability of the EAH to inhibit lipid peroxidation was time-dependent and follows a similar trend to that of ellagic acid.

The swelling behaviour of hydrogel were examined at two different pHs, 6.2, simulating the pathological cancerous environment, and 7.4 the effective physiological pH. The values were examined at different time-points (1, 6, 12 and 24 h). Each experiment was carried out in triplicate and the results

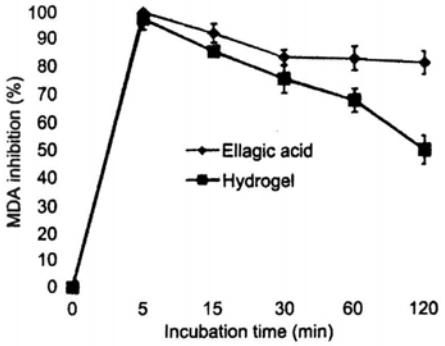

Figure 6. *Anti-oxidant activity evaluation. MDA: Malondialdehyde. Data are expressed as the mean±S.D. of at least three independent experiments.*

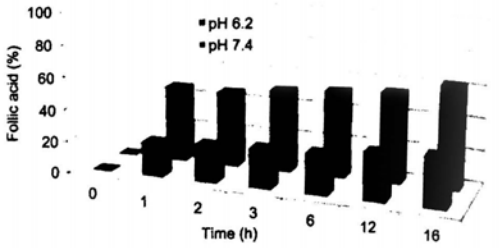

Figure 7. *In vitro release studies. Each experiment was carried out in triplicate and the results were in agreement within ±4% standard error.*

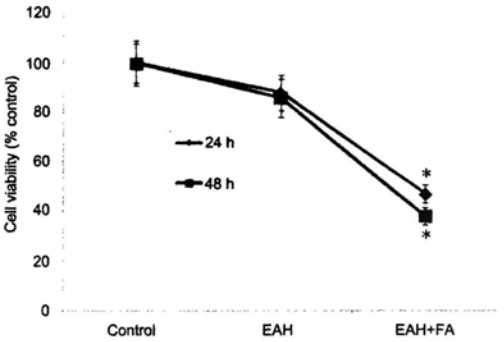

Figure 8. *Effect of formulation on MCF-7 cell viability. *p<0.05 compared to control. Data are expressed as the mean±S.D. of at least three independent experiments.*

were in agreement within ±4% standard error. The swelling percent (α%) for the prepared material is reported in Table I.

The FA was entrapped into the preformed EAH by a soaking procedure consisting in the dissolution of FA in a solution that allows the swelling of polymeric matrix. Loading efficiency was calculated by UV-Vis spectrophotometric analysis of the filtered solution (λ=365 nm, =7595 $mol^{-1}dm^3cm^{-1}$). The experiment was repeated in triplicate and results showed that the drug was almost completely loaded into the matrix or on its surface. The entrapment efficiency was 75%±1.8.

The release studies of FA were conducted on aliquots of preformed hydrogel at two different pHs (6.2 and 7.4) and at different time-points (1, 6, 12 h and 24 h) by using a shaking water bath at 37°C (body temperature). The results showed that FA was characterized by a slow kinetic-release, especially at pH 6.2 (Figure 7).

The cytotoxic effect of EAH on MCF-7 cells was evaluated. The results of this study demonstrated that the addition of EAH (50 μM) alone to MCF-7 cells for 24 and 48 h did not lead to statistically significant cytotoxic effects as compared with untreated cells (Figure 8). In addition, in order to evaluate the anti-tumoral effect of EAH+FA, an MTT assay was carried out in MCF-7 cells exposed to the polymeric system. As shown in Figure 8, a significant reduction (*$p<0.05$ *vs.* control) of viability was observed in the cells exposed for 24 and 48 h to 50 μM EAH+FA (Figure 8). Notably, EAH+FA dramatically increased anti-tumoral activity, inducing 53% cell death *vs.* control at 24 h, and actually reaching 62% suppression of viability after 48 h. Similar data were obtained in MDA-MB-231 cells (data not shown). These results suggest that the utilization of the polymeric system EAH+FA for localized breast cancer therapy may have a high potential for success.

Table I. *Swelling behavior of hydrogel.*

Time (h)	Swelling (α%)	
	pH 6.2	pH 7.4
1	125	482
6	224	712
12	252	752
24	277	771

α% was calculated as

$$\alpha\% = \frac{(Ws - Wd)}{Wd} \times 100$$

where Ws and Wd are weights of swollen and dried hydrogel, respectively.

Conclusion

The aim of the present work was the realization of a biocompatible hydrogel, based on ellagic acid and glycine, carrying FA, a model molecule, useful in subcutaneous implant in breast cancer treatment. The results confirmed the possibility that polymeric systems based on natural substances may find use in the treatment of cancer, where conventional therapy provides the onset of serious side-effects. The degree of swelling of the hydrogel not containing FA was studied at two different pH values and at fixed time intervals. In particular, we used phosphate buffers at pH 6.2 to mimic conditions typical of tumor pathology, and pH 7.4 to simulate the physiological environment. The obtained results showed that this hydrogel swelled more at pH 7.4. This could be attributed to the numerous phenolic groups, which being predominantly in their ionic form, increased the hydrophilicity of the ellagic acid hydrogel. This latter, loaded with FA, was also subjected to preliminary release studies conducted at pH 6.2 and pH 7.4. These studies have shown that FA was released similarly from the matrix irrespective of pH. More precisely, the release occurred slowly during the first 6 h and then increased gradually, probably due to the greater swelling of the hydrogel. In particular, FA was released over time in a relatively small quantity, therefore, it can be concluded that more drug could be released after 24 h. It is also interesting to note that FA was characterized by a slow kinetic release, especially at pH 6.2, typical of the cancerous environment. In addition, *in vitro* studies demonstrated that EAH containing FA, significantly inhibited the growth of human breast cancer cells. Our results show how EAH+FA exerted a rapid (as soon as after 24 h of treatment) and sustained cytotoxic effect, causing almost 62% of cell death within 48 h of exposure, if compared to EAH-alone, which was only able to exert a cytostatic effect. Finally, studies aimed at assessing antioxidant activity of the hydrogel not containing FA have shown the preservation of ellagic acid behavior. These studies were performed by monitoring the levels of malondialdehyde in rat liver microsomal membranes and revealed the ability of the hydrogel (EAH) to inhibit lipid peroxidation following the same trends of ellagic acid.

In conclusion, on the basis of the obtained results we can hypothesize a possible use of EAH as a subcutaneous implant for controlled and site-specific release of FA useful in the treatment of breast cancer.

Acknowledgements

This work was financially supported by MIUR (Programma di Ricerca di Rilevante Interesse Nazionale 2008) and University of Calabria funds. This work was also supported by Associazione Italiana Ricerca sul Cancro (AIRC) [MFAG grant number 6180 to Loredana Mauro and IG grant number 11595 to Sebastiano Andò] and by European Commission/FSE/Regione Calabria to Emilia Ricchio. The Authors are solely responsible for the work.

References

1 Cassano R, Trombino S, Ferrarelli T, Bilia AR, Bergonzi MC, Russo A, De Amicis F and Picci N: Preparation, characterization and *in vitro* activities evaluation of curcumin based microspheres for azathioprine oral delivery. React Funct Polym *72*: 446-450, 2012.

2 Trombino S, Cassano R, Ferrarelli T, Isacchi B, Bilia AR and Picci N: Collagen α-tocopherulate for topical applications: Preparation, characterization and antioxidant activity evaluation. Macromol Res *20*: 939-943, 2012.

3 Trombino S, Cassano R, Ferrarelli T, Cavalcanti P, Giraldi C, Lai F, Loy G and Picci N: Synthesis and *in vitro* antitubercular activity of isoniazid-gelatin conjugate. J Pharm Pharmacol *64*: 712-718, 2012.

4 Trombino S, Cassano R, Ferrarelli T, Mauro MV, Giraldi C, Manconi M, Fadda AM and Picci N: Respirable rifampicin-based microspheres containing isoniazid for tuberculosis treatment. J Biomed Mater Res Part A *100A*: 536, 2012.

5 Langer R: Biomaterials in drug delivery and tissue engineering: One laboratory's experience. Acc Chem Res *33*: 94-101, 2000.

6 Lu Y and Chen SC: Micro and nano-fabrication of biodegradable polymers for drug delivery. Adv Drug Deliv Rev *56*: 1621-1633, 2004.

7 Hoare T and Kohane DS: Hydrogels in drug delivery: Progress and challenges. Polymer *49*: 1993-2007, 2008.

8 Qiu Y and Park K: Environment-sensitive hydrogels for drug delivery. Adv Drug Deliv Rev *64*: 49-60, 2012.

9 Cassano R, Trombino S, Muzzalupo R, Tavano L and Picci N: A novel dextran hydrogel linking *trans*-ferulic acid for the stabilization and transdermal delivery of vitamin E. Eur J Pharm Biopharm *72*: 232-238, 2009.

10 Trombino S, Cassano R, Bloise E, Muzzalupo R, Tavano L and Picci N: Synthesis and antioxidant activity evaluation of a novel cellulose hydrogel containing trans-ferulic acid. Carbohydr Polymer *75*: 184-188, 2009.

11 Talcott ST and Joon-Hee L: Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. J Agric Food Chem *50*: 3186-3192, 2002.

12 Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG and Heber D: *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nut Biochem *16*: 360-367, 2005.

13 Festa F, Aglitti T, Duranti G, Ricordi R, Perticone P and Cozzi R: Strong antioxidant activity of ellagic acid in mammalian cells *in vitro* revealed by the comet assay. Anticancer Res *21*: 3903-3908, 2001.

14 Yu YM, Chang WC, Wu CH and Chiang SY: Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid. J Nutr Biochem *16*: 675-681, 2005.

15 Stoner GD and Gupta A: Etiology and chemoprevention of esophageal squamous cell carcinoma. Carcinogenesis *22*: 1737-1746, 2001.

16 Larrosa M, Tomas-Barberan FA and Espin JC: The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J Nutr Biochem *17*: 611-625, 2006.

17 Dorai T and Aggarwal BB: Role of chemopreventive agents in cancer therapy. Cancer Lett *215*: 129-140, 2004.

18 Han DH, Lee MJ and Jeong HK: Antioxidant and apoptosis-inducing activities of ellagic acid. Anticancer Res *26*: 3601-3606, 2006.

19 Lucock M: Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. Mol Gen Metab *71*: 121-138, 2000.

20 John F, Ross BS, Prabir K, Chaudhuri MD and Ratnam M: Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines. Physiologic and clinical implications. Cancer *73*: 2432-2443, 1994.

21 Guo M, Que C, Wang C, Liu X, Yan H and Liu K: Multifunctional superparamagnetic nanocarriers with folate-mediated and pH-responsive targeting properties for anticancer drug delivery. Biomaterials *32*: 185-194, 2011.

22 Barlos K, Papaioannou D and Theodoropoulos D: Efficient "one-pot" synthesis of *N*-trityl amino acids. J Org Chem *47*: 1324-1326, 1982.

23 Vanhaecht B, Teerenstra MN, Suwier DR and Koning CE: Dicyclohexylcarbodiimide assisted synthesis of aliphatic polyesters at room temperature. J Macromol Sci Part A: Pure Appl Chem *37*: 633-643, 2000.

24 Theodorou V, Ragoussis V, Strongilos A, Zelepos E, Eleftheriou A and Dinitriou M: A convenient method for the preparation of primary amines using tritylamine. Tetrahedron Lett *46*: 1357-1360, 2005.

25 Trombino S, Cassano R, Bloise E, Muzzalupo R, Leta S, Puoci F and Picci N: Design and synthesis of cellulose derivatives with antioxidant activity. Macromol Biosci *8*: 86-95, 2008.

26 Trombino S, Serini S, Di Nicuolo F, Celleno L, Andò S, Picci N, Calviello G and Palozza P: Antioxidant effect of ferulic acid in isolated membranes and intact cells: synergistic interactions with alpha-tocopherol, beta-carotene, and ascorbic acid. J Agric Food Chem *52*: 2411-2420, 2004.

27 Trombino S, Cassano R, Muzzalupo R, Pingitore A, Cione E and Picci N: Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of beta-carotene and alpha-tocopherol. Coll Surf B: Biointer *72*: 181-187, 2009.

28 Panno ML, Giordano F, Rizza P, Pellegrino M, Zito D, Giordano C, Mauro L, Catalano S, Aquila S, Sisci D, De Amicis F, Vivacqua A, Fuqua FW and Andò S: Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination. Breast Cancer Res Treat *136*: 443-455, 2012.

Received August 5, 2013
Revised September 27, 2013
Accepted September 30, 2013

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 Ricchio Emilia. L'attività di ricerca svolta dalla Dott.ssa **Ricchio** è stata rivolta principalmente allo studio del ruolo dei fattori del microambiente tumorale sulla progressione del carcinoma mammario umano in soggetti obesi. In particolare la ricerca è stata finalizzata alla comprensione dei meccanismi molecolari attivati dall'adiponectina, la più abbondante adipochina secreta dal tessuto adiposo bianco, e coinvolti nei processi proliferativi di cellule di carcinoma mammario umano. I risultati ottenuti mediante studi *in vitro* durante i primi due anni di Dottorato sono stati ulteriormente approfonditi generando un modello xenograft impiantando cellule di carcinoma mammario umano in topi atimici.
Da Aprile ad Ottobre 2014 la Dott.ssa Ricchio ha svolto la sua attività di ricerca presso il laboratorio diretto dal Dr. Michael Lisanti presso il Breakthrough Breast Cancer Research Unit, University of Manchester. Durante lo stage la Dott.ssa Ricchio è stata coinvolta nella realizzazione di un progetto finalizzato a valutare il ruolo dei fibroblasti stromali nello sviluppo dell'ormono-resistenza e staminalità in cellule di tumore mammario esprimenti il recettore estrogenico.

La Dott.ssa Ricchio ha mostrato 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. Si esprime pertanto parere estremamente positivo sul lavoro svolto durante il terzo anno di dottorato di ricerca.

La tematica di ricerca svolta dalla Dott.ssa Ricchio è coerente con il polo di innovazione "Tecnologie della Salute" assegnato in fase concorsuale. La dottoranda ha svolto regolarmente le attività di studio e di ricerca previste per il terzo anno di corso e, pertanto, può essere ammessa a sostenere l'esame finale.

Il Collegio dei docenti valutato il contenuto della relazione di fine anno della Dott.ssa Emilia Ricchio 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 Coordinatore
Prof. Diego Sisci