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Modulatory role of adiponectin on endocrine resistance in breast cancer

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Abstract

Estrogen-Receptor (ER) represents a breast cancer positive prognostic factor and the main molecular target of endocrine therapy, although *de novo* and acquired resistance to this treatment remains a major challenge. Breast Cancer Stem Cells (BCSCs) are blamed to be the driving force behind breast tumor initiation, progression, metastasis, endocrine resistance, and recurrence, due to their strong self-renewal and multi-lineage differentiation properties. Notably, it has been evidenced that tumor microenvironment (TME) influences BCSCs behavior, regulating the complex interaction between stromal and breast epithelial cells, through the secretion of different cytokines and growth factors. The most abundant cellular component of TME is represented by adipocytes which became dysfunctional in obesity, leading to an unbalanced adipokines secretion. Particularly, plasma levels of adiponectin, the main adipocyte-derived factor, are reduced in obese compared to normal weight subjects, promoting growth and progression in ERα-positive breast cancer. This let us to wonder if the induced proliferative effect of low level of adiponectin on ERα-positive breast cancer cells could be related to specific stimulatory effects on breast cancer stemness. In tamoxifen-resistant MCF-7 (TR MCF-7) mammospheres adiponectin increased Mammospheres Forming Efficiency (MFE) and self-renewal capacity concomitant with an enrichment in $CD44^+/CD24^-/ALDH1^+$ subpopulation, identifying a typical stem-like phenotype. This was not observed in hormone-responsive MCF-7 (WT MCF-7) cells. An increase in mRNA levels of stemness and Epithelial-Mesenchymal Transition (EMT) markers corroborated the increased percentage of BCSCs in TR compared to WT MCF-7 mammospheres. Consistent with these data

hormone-resistant cells showed a G0/G1 arrest and an apoptosis reduction compared to WT cells, as evidenced by cell cycle and Annexin V assay, Western Blotting and proteomic analysis. Interestingly, in adiponectin-treated TR MCF-7 mammospheres, it has been observed a reduced ROS production, concomitant with a slight enhance of mitochondrial membrane potential, confirming the anti-apoptotic features of BCSCs. Finally, TR MCF-7 cells, obtained from secondary generation mammospheres, exhibited a reduced proliferation rate, Ki67 level and cell motility, suggesting a cellular state of quiescence in TR MCF-7 cells.

Overall, we demonstrated that low levels of adiponectin promote stem-like features in TR MCF-7 cells, that may contribute to relapse.

Thus, the study of the molecular mechanisms involved in the regulation of cell death and in the entry of a quiescent state, could pave the way for the development of successful therapies for the treatment of hormone-resistant obese breast cancer patients.

Riassunto

Il recettore estrogenico (ER), fattore prognostico positivo per il carcinoma mammario, si configura come il principale bersaglio molecolare per il trattamento basato sulla terapia ormonale. Tuttavia, la resistenza endocrina intrinseca (*de novo*) e la resistenza acquisita costituiscono un'importante sfida clinica nel trattamento della patologia. Le cellule staminali del cancro al seno, caratterizzate dalla capacità di autorinnovamento e di differenziazione in vari tipi cellulari, sono ritenute responsabili dell'insorgenza e della progressione tumorale, del processo metastatico, della resistenza endocrina e dei meccanismi di recidiva. In particolare, è stato evidenziato che il microambiente tumorale influenza il comportamento delle cellule staminali del tumore mammario, regolando la complessa interazione tra le cellule epiteliali mammarie e stromali attraverso la secrezione di diverse citochine e fattori di crescita. La componente cellulare più abbondante del microambiente tumorale è rappresentata dagli adipociti, i quali secernono livelli alterati di adipochine in condizioni di obesità. In particolare, i livelli plasmatici di adiponectina, principale adipochina prodotta, sono ridotti nei soggetti obesi rispetto a quelli normopeso, favorendo la crescita e la progressione nel cancro al seno ERα-positivo. Pertanto, è stato valutato se l'effetto proliferativo, indotto da bassi livelli di adiponectina sulle cellule di carcinoma mammario ERα-positive, potesse essere correlato allo sviluppo di un fenotipo staminale. Nelle mammosfere ottenute da cellule di carcinoma mammario MCF-7 resistenti al tamoxifene (TR MCF-7), l'adiponectina ha indotto un aumento della capacità di formazione delle mammosfere e di autorinnovamento e un arricchimento della sottopopolazione $CD44^{+}/CD24^{-}/ALDH1^{+}$, identificando un fenotipo simil-staminale. Ciò non è stato

osservato nelle cellule MCF-7 ormono-responsive (WT MCF-7). Un aumento dei livelli di espressione genica dei marker di staminalità e dei marker della transizione epitelio-mesenchimale ha confermato l'aumento della percentuale di cellule staminali tumorali mammarie nelle mammosfere ottenute da cellule TR MCF-7, rispetto a quelle ottenute da cellule WT MCF-7. Coerentemente, le cellule TR MCF-7 hanno mostrato arresto del ciclo cellulare in fase G0/G1 e riduzione dell'apoptosi rispetto alle cellule WT, come evidenziato dal ciclo cellulare e dal test dell'Annessina V, dal Western Blotting e dall'analisi proteomica. È interessante notare che nelle mammosfere ottenute a partire da cellule TR MCF-7 e trattate con adiponectina è stata osservata una ridotta produzione delle specie reattive dell'ossigeno (ROS) concomitante con un lieve aumento del potenziale di membrana mitocondriale, confermando che le cellule staminali del cancro al seno tendono a sfuggire ai meccanismi apoptotici. Infine, le cellule TR MCF-7 ottenute da mammosfere di seconda generazione mostrano un tasso proliferativo ridotto, bassi livelli di Ki67 e una minore motilità cellulare, suggerendo l'insorgenza di uno stato di quiescenza.

Nel complesso, è stato dimostrato che bassi livelli di adiponectina promuovono nelle cellule TR MCF-7 l'insorgenza di un fenotipo simil-staminale che potrebbe svolgere un ruolo chiave nell'ambito della recidiva. Pertanto, lo studio dei meccanismi molecolari coinvolti nella regolazione della morte cellulare e nell'insorgenza di uno stato quiescente potrebbe essere utile per lo sviluppo di nuove terapie per il trattamento delle pazienti obese affette da tumore mammario ormono-resistente.

Introduction

1. Breast cancer and obesity

Breast cancer is the most commonly diagnosed malignancy in women, accounting for 24.5% of total cancer cases and 15.5% of cancer related deaths worldwide[1]. Although the incidence rates of breast cancer is steadily increasing, early diagnosis and effective treatment have led to a significant improvement of the overall 5-year survival rate for mammary tumor, reaching the 91% from 63% in the early 1960s[2]. Nevertheless, the 5-year survival rate is still arrested at just 26% for patients with advanced or metastatic breast cancer at the time of diagnosis[3].

Breast cancer is a heterogeneous disease that include a wide range of tumors with diversified morphology, gene expression patterns, clinical phenotype and prognosis[4]. Accurate and precise tumor classification is required to obtain an appropriate diagnosis and careful prediction of cancer behaviour to define a tailored therapeutic approach[4,5].

Histological characterization of the neoplastic mass is the primary step to follow in order to acquire detailed diagnostic and prognostic indications[6]. In particular, to identify a histological subtype is necessary to consider several criteria encompassing tumor cell type, extracellular secretion, cell architecture characteristics and immunohistochemical pattern[5].

Infiltrating ductal carcinoma or IDC is the most common histological form, which represents around 80% of all invasive breast cancer, followed by invasive lobular carcinoma (ILC) accounting for approximately 10% of all invasive mammary tumors. The remaining part consists of the less common histologic types, including

mucinous, cribriform, micropapillary, papillary, tubular, medullary, metaplastic, and apocrine carcinomas[5].

Another notable parameter used as powerful prognostic factor is histological grade resulting from microscopic assessment of tumor differentiation in terms of tubule formation, nuclear pleomorphism and proliferation rate evaluated as mitotic index. Nottingham Modification of the Bloom-Richardson system rates each of the three parameters from 1 to 3 and provides an overall summation score[5] (Figure 1).

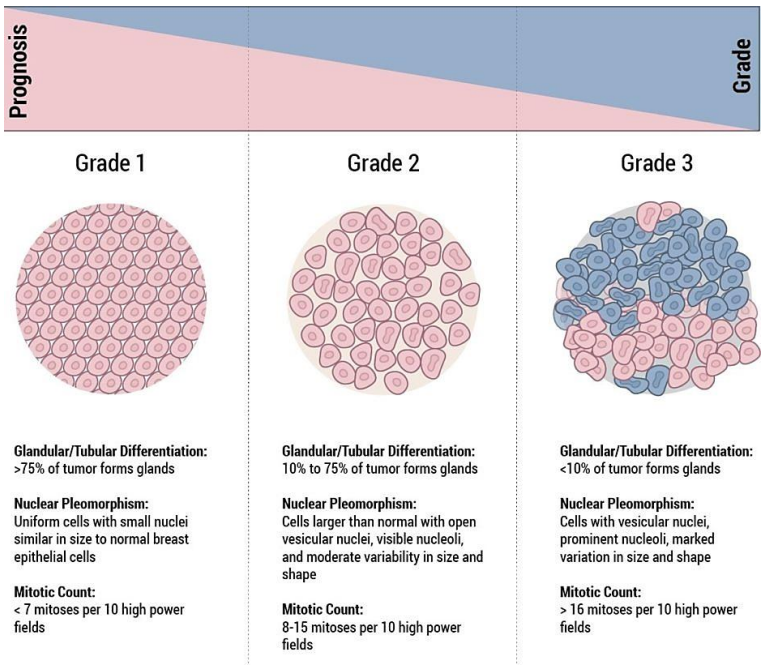


Figure 1. Breast cancer grade

However, traditional breast cancer classification also exploits TNM system as prognostic and disease staging factor. Through the evaluation of the clinical-pathological pattern, TNM staging provides information about tumor size (T), involvement of regional lymph nodes (N) and distant metastasis (M). Specifically,

this staging system allows the neoplastic disease to be classified into one of 5 stages (0, I, II, III, and IV)[5] (Table 1).

Table 1. TNM system

		Stage	Primary tumour (T)*	Regional lymph node status (L)	Distant metastasis (M)
T- Tumour		**0**	Tis	N0	M0
T1	Tumour ≤ 2 cm	**I**	T1	N0	M0
T2	Tumour ≥ 2 cm but ≥ 5 cm		T0	N1	M0
T3	Tumour ≥ 5 cm	**IIA**	T1	N1	M0
T4	Tumour of any size with direct extension to chest wall or skin		T2	N0	M0
N- Lymph node		**IIB**	T2	N1	M0
N0	No cancer in regional node		T3	N0	M0
N1	Regional movable metastasis	**III A**	T0	N2	M0
N2	Non-movable regional metastases		T1	N2	M0
N3	Cancer in the internal mammary lymph nodes		T2	N2	M0
M- Metastasis			T3	N1/N2	M0
M0	No distant metastases	**III B**	T4	Any N	M0
M1	Distant metastases	**III C**	Any T	N3	M0
		IV	Any T	Any N	M1

Criteria for staging breast tumours according to the UICC ICD-10 TNM classification.

*Size measurements are for the tumour's greatest dimension.

Finally, according to the current molecular classification, breast cancers are grouped as follows: luminal A: ER+ and/or PR+, HER2- and low Ki67 (<14%); luminal B: ER+ and/or PR+, HER2+ or HER2- and high Ki67 (>14%); HER2+: ER-, PR- and HER2+; and basal-like (BLBC): ER-, PR-, HER2- (triple negative), plus CK 5/6+ and/or EGFR+[4].

However, it is worth noting that the complex heterogeneity of breast cancer may also result from the influence of several adverse factors that may regulate tumor progression and development[7].

Family history is one of the major risk factors for breast cancer. Although sundry genetic mutations enhance breast cancer incidence rate, roughly 40% of inherited mammary tumors are caused by autosomal dominant mutations in BRCA1 and BRCA2 tumor suppressor genes. Aside from genetic and hereditary influence, breast cancer pathogenesis is more commonly associated with a myriad of other factors including demographic, reproductive, hormonal, environmental, and lifestyle factors, some of which may be potentially modifiable[8,9].

Among modifiable factors, several studies have evidenced the high correlation between obesity and breast cancer risk, highlighting significant differences in breast cancer recurrence and mortality according to menopausal status and mammary tumor subtypes[10]. In particular, obesity is a chronic and multifactorial disorder that can seriously compromise human health. It is caused by excessive fat tissue accumulation due to both pre-existing adipocytes hypertrophy and *de novo* adipocytes hyperplasia. The World Health Organization (WHO) and the National Institutes of Health (NIH) refer to obesity as a body mass index greater than 30 kg/m^2 (30.0-34.9, grade I; 35.0-39.9, grade II; and ≥40, grade III)[10,11]. The complexity of the obesity condition and the several oncogenic alterations, that may support breast cancer tumorigenesis, make the knowledge of the molecular mechanisms linking breast cancer development and obesity still under investigation[11]. Surely, a crucial mechanism through which obesity may sustain the malignant transformation of breast tissue, especially in postmenopausal women, is represented by an increase in circulating estrogen levels consequent to a greater aromatase activity responsible for androgens conversion[12]. In this context, 17β-estradiol (E2) binding to estrogen receptor alpha (ERα) can trigger the activation of

genomic (classical or non-classical) and non-genomic actions in order to regulate the expression levels of downstream proteins involved in cell proliferation, survival and invasion[13].

Many experimental and epidemiological supports show that the association between high body weight and breast cancer risk and prognosis is regulated not only by estrogen activity but also by several estrogen-independent mechanisms[14] (Figure 2).

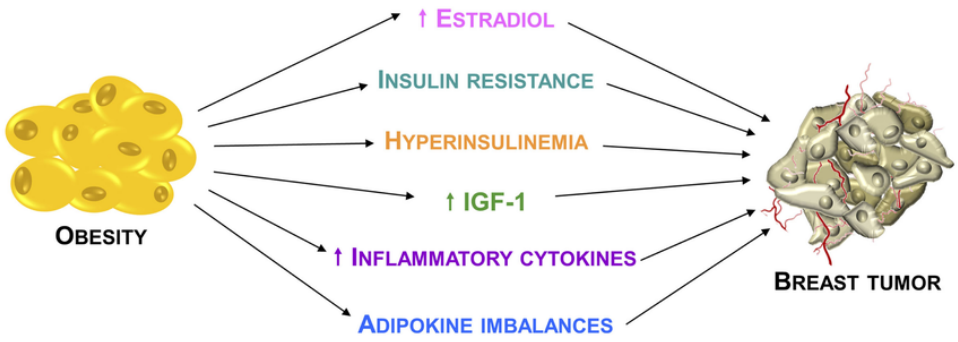


Figure 2. Relationship between obesity and breast cancer

Indeed, it has been reported that insulin resistance, hyperinsulinemia, and/or hyperglycemia are considered hallmarks of obese condition that indirectly may contribute to breast cancer development. Particularly, insulin promotes carcinogenesis through a direct effect on insulin receptors or an indirect effect by regulating the levels of other modulators, such as the insulin-like growth factor (IGF) family of receptors. IGF-1, one of the most important growth factors released by adipocytes, stimulates their differentiation and metabolic regulation. Moreover, its binding to IGF-1R, a tyrosine kinase receptor, induces the activation of Phosphoinositide 3-kinase (PI3K)/Protein kinase B (Akt) and Mitogen-activated

protein kinase (MAPK) signaling, which alters the expression of genes involved in cellular proliferation and survival[15].

In addition, the interaction between tumor cells and the surrounding tumor microenvironment (TME), comprising stromal cells, soluble factors, signaling molecules, and extracellular matrix, is another important mechanism involved in obesity-mediated breast carcinogenesis[14]. Adipocytes are key components in the stroma of breast carcinoma and, in obese condition, through their metabolic substrates, such as both saturated and unsaturated fatty acids (FAs) and matrix metalloproteinase (MMPs), can activate signaling that contribute to create a low chronic inflammatory status of TME. These events induce the recruitment of macrophages that encircle damaged, dying or dead adipocytes forming a crown-like structure (CLS) in adipose stromal breast tissue. Macrophage CLSs negatively influence breast cancer recurrence and survival[16,17]. Adipocytes may stimulate chronic inflammation, tumor initiation and metastatic burden in breast cancer also through the release of several cytokines such as interleukin 6 (IL-6), interleukin 8 (IL-8) and IFNγ-inducible protein 10 (CXCL10), chemokines (CCL2 and CCL5) and a large group of heterogeneous peptides named as adipokines (Figure 3).

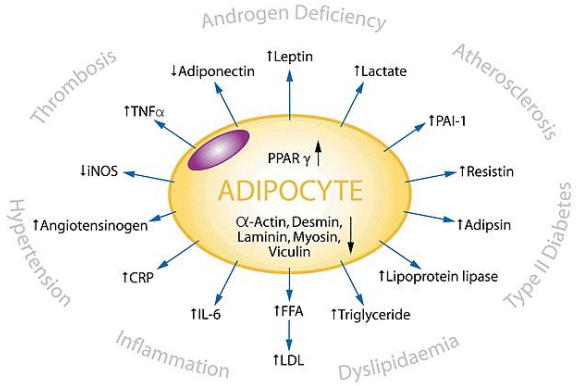


Figure 3. Bioactive molecules secreted by adipocytes

Changes in adipokine profile, due to the altered fat distribution and function, is one of the main features in obese state. Currently, more than 100 different adipokines have been identified, and among these, leptin and adiponectin are recognized for their impact on breast cancer risk and tumor biology.

2. Adiponectin

2.1 Adiponectin structure and receptors

Adiponectin (also known as *Acrp30*[18], *AdipoQ*[19], *GBP-28*[20] , and *apM1*[21]) is one of the most important members of the adipocytokine family[22]. It is mainly secreted by white adipose tissue and, as subsequently reported by several research groups, it is also expressed, to a limited extent, in other tissues, including human and murine osteoblasts[23], liver parenchyma cells[24], myocytes[25], epithelial cells[26], and placental tissue[27].

Human adiponectin is encoded by the *Adipo Q* gene (16 kb), which maps to chromosome locus 3q27, a region associated with susceptibility for developing metabolic syndrome and type 2 diabetes in Caucasians[28]. It contains three exons, with the start codon in exon 2 and stop codon in exon 3. Many reports showed several single nucleotide polymorphisms (SNPs) in the coding region associated with alterations of adiponectin function and important clinical conditions. In particular, SNPs enhance adiponectin effects on insulin resistance, type 2 diabetes, obesity, dyslipidemia, and many obesity-related malignancies[29]. Structurally, adiponectin is a 30 kDa glycoprotein consisting of 244 amino acids organized into four domains: an amino-terminal signal peptide represented by 18 amino acids, a

hyper-variable region of 28 amino acids, a collagen-like motif containing 22 Gly-X-Y repeats, and a 137-amino acid carboxy-terminal globular domain important for the interaction with the receptor[21,30,31] (Figure 4).

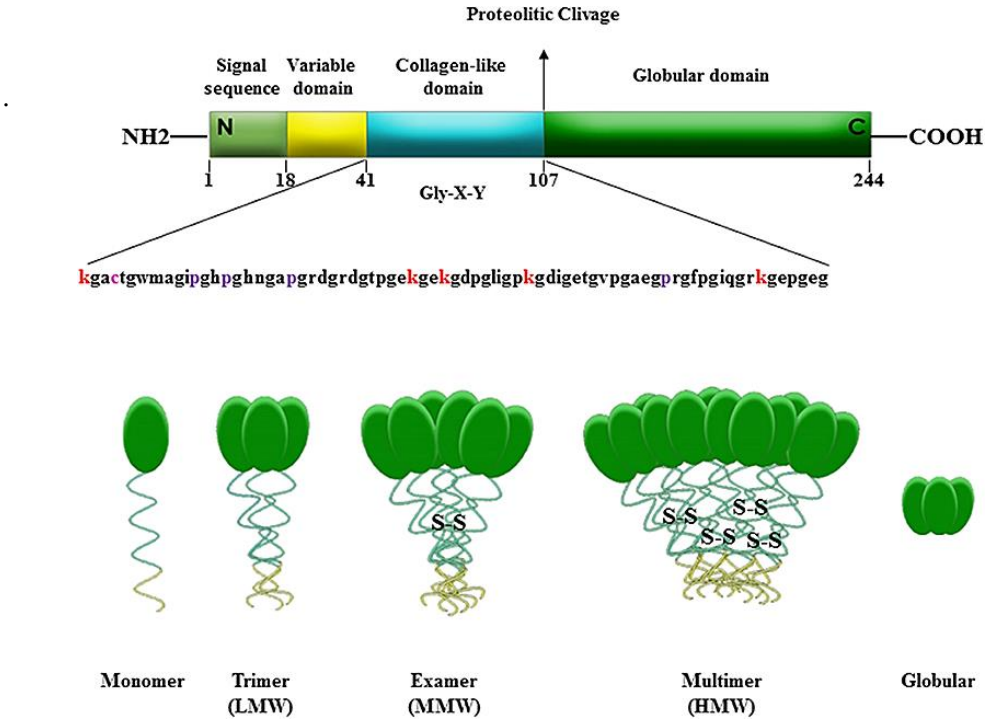


Figure 4. Domains and molecular structure of adiponectin

Once synthesized, the monomeric form of adiponectin undergoes to post-translational modifications, like hydroxylation and glycosylation, that allow the formation of disulfide-linked oligomers composed of trimers (Low Molecular Weight, LMW), hexamers (Medium Molecular Weight, MMW), and multimers (High Molecular Weight, HMW), detectable in the circulation[32,33]. Moreover, in human plasma adiponectin exists also as a proteolytic cleavage fragment (globular adiponectin, gAd), produced by leukocyte elastase activity on amino acid position 110 of the full-length protein (fAd)[34,35]. Several studies have revealed that the different adiponectin isoforms have various target tissues and can induce different

biological responses[36]. The HMW isoform represents the biologically active form of the hormone and exerts most of adiponectin effects in the liver[37], endothelial cells[38], and probably in skeletal muscle[39]. In particular, it plays a key role in the regulation of insulin resistance, metabolic syndrome, and cardiovascular disease and it acts as pro-inflammatory cytokine. Conversely, LMW isoform is present at low plasma concentration, probably due to its shorter half-life, and it is responsible for anti-inflammatory actions[32,34].

As it concerns physiology, adiponectin represents approximately 0.01% of the total human plasma proteins, with concentrations ranging from 3 to 30 μg/ml, depending on hormonal, inflammatory, pharmacological and dietary factors[31,36]. Unlike most of the other adipose tissue-derived proteins, adiponectin plasma levels are reduced in obese compared to normal-weight subjects, correlating negatively with body mass index (BMI). The mechanisms underlying the down-regulation of this adipokine have not yet been fully elucidated. However, many evidence suggests that the chronic low inflammatory state of adipose tissue and the adipocytes dysfunction that characterize obesity condition may stimulate hypoadiponectinemia. In particular, it has been demonstrated that the reduced adiponectin levels in obesity may be caused by the increased production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6)[14,40]. Another potential mechanism involved could be represented by a negative feedback of adiponectin on its own production and probably on the expression of its receptors during obesity development[36]. Also, association-studies correlate different SNPs in the gene encoding adiponectin with reduced levels of this adipokine[29,41]. In addition, the generation of a hypoxic microenvironment in

hypertrophic adipocytes and an increased production of insulin-like growth factor binding protein-3 (IGFBP-3), which inhibits adiponectin transcription via hypoxia inducible factor-1α (HIF-1α) dependent pathway, could further explain adiponectin down-regulation[36]. Finally, it has been well documented the involvement of several hormonal factors, such as prolactin, growth hormone (GH), glucocorticoids[42] and testosterone[43], in the negative regulation of adiponectin expression.

Adiponectin actions are mediated by its binding to three distinct membrane receptors: AdipoR1, AdipoR2 and T-cadherin[44].

AdipoR1 and AdipoR2, classical adiponectin receptors, are seven-transmembrane proteins that show structural and functional similarity, but opposite topology, to G protein-coupled receptors (GPCRs); specifically, unlike GPCRs, adiponectin receptors exhibit an internal N-terminus domain and an external C-terminus domain[45]. Both receptor isoforms are detected in nearly all tissues, although the expression ratio is generally different. Indeed, AdipoR1 is abundant in skeletal muscle and endothelial cells, while AdipoR2 is mainly expressed in the hepatocytes[46]. Despite a protein sequence homology of approximately 67%, the two classical adiponectin receptors show dissimilar affinity for the adipokine oligomers. In particular, AdipoR1 is characterized by high affinity for gAd and low affinity for the fAd[47], while AdipoR2 has intermediate affinity for both isoforms of adiponectin[46].

The third non-classical adiponectin receptor, T-cadherin, is a glycosyl-phosphatidylinositol-anchored extracellular protein mainly detected in endothelial cells, smooth muscle cells and in cardio myocytes[48]. It shows affinity for MMW and HMW but not for trimeric and globular forms of adiponectin[49,50] (Figure 5).

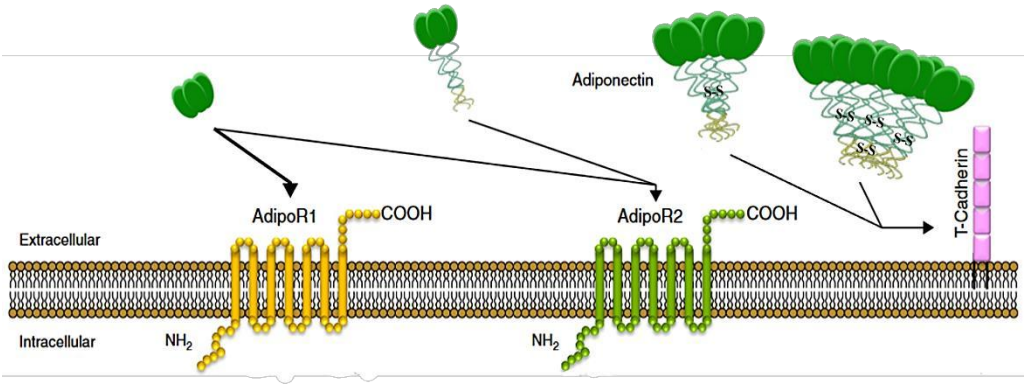


Figure 5. Affinity of adiponectin isoforms for the different receptors

Many studies evidenced the involvement of T-cadherin in cell adhesion and in calcium-mediated cell to cell interactions and signaling[48]. Moreover, it is well recognized that, contrariwise to AdipoR1 and AdipoR2, T-cadherin is a cell-surface receptor lacking transmembrane domain, crucial for signal transduction; therefore, it has been suggested that it may function as a co-receptor, competing with AdipoR1 and AdipoR2 receptors for adiponectin binding or interfering with adiponectin signal transduction[51].

Several factors can modulate the expression of adiponectin receptors. It has been reported a direct correlation between the expression levels of adiponectin receptors and the tissue expression levels of adiponectin itself in adipose tissue[52]. Indeed, the expression of AdipoR1/R2 seems to be decreased in obesity, thereby diminishing adiponectin sensitivity, which in turn leads to a vicious cycle of insulin resistance[53]. In addition, many studies evidenced that adiponectin is able to up-regulate AdipoR1 receptor, but not AdipoR2, in differentiated primary skeletal muscle cells from normal-weight subjects; however, this effect was not observed in diabetic and obese patients and in those who lost weight[54]. Although the expression of adiponectin

receptors in adipose tissue is not affected by sexual dimorphism[55], experimental evidence showed that AdipoR1/R2 expression is associated with sex in muscle, with a high expression in male subject[56]. AdipoR1 and AdipoR2 expression levels are also associated with age and aerobic capacity in skeletal muscle[56]. Interestingly, adiponectin receptors are expressed in a plethora of malignant tissues including breast[57], prostate[58], hepatocellular, gastric, colon[59], lung cancer[60,61] and pancreatic adenocarcinoma[62]. Although the functional relevance of adiponectin receptors in cancerous cells has not yet been clarified, there is evidence that activation of adiponectin receptors limits the proliferation of cancer cell lines *in vitro*[63].

2.2 Adiponectin functions

Adiponectin exerts pleiotropic actions on different organs and tissues. Interestingly, it has insulin-sensitizing, antiatherogenic, anti-inflammatory, cardioprotective properties, as well as distinct effects on lipid metabolism[63,64] (Figure 6).

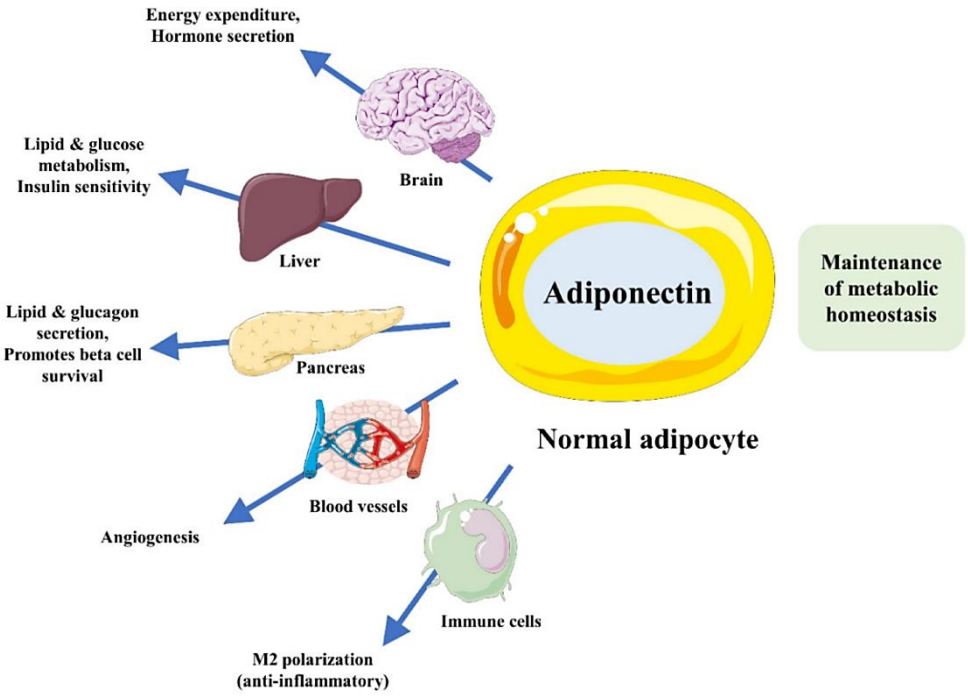


Figure 6. Adiponectin actions on different organs and tissue

Injection of recombinant adiponectin has been shown to enhance circulating insulin levels in mice[65]. Indeed, adiponectin treatment of pancreatic β cells stimulates the up-regulation of *PDX-1* and *MafA* genes, responsible for the co-activation of insulin gene, resulting in increased insulin exocytosis[66]. Moreover, it is known the ability of adiponectin to enhance insulin-induced phosphorylation of the insulin receptor (IR) and insulin capability to activate the phosphorylation of the adaptor protein insulin receptor substrate 1 (IRS-1)[67]. Adiponectin may also promote insulin-sensitizing actions regulating lipid metabolism. It acts redirecting fatty acids to the muscles for their oxidation and decreasing the influx of fatty acids and the total triglyceride content to the liver[68,69]. In particular, this adipokine affects triglyceride reduction by supporting increased expression of protein involved in fatty acid transport, fatty acid combustion mediated by Acetyl-coenzyme A carboxylase (ACC) and energy dissipation by uncoupling protein (UCP) 2[68,70]. However, an

opposite trend is observed in adipocytes, where this adipokine favours triglycerides accumulation, stimulating the storage function of adipose tissue[71].

In addition, adiponectin also regulates the transcription of many genes involved in lipid catabolism, such as Acetyl-CoA Oxidase (ACO), Fatty-acid binding-protein 3 (FABP3), and Carnitine Palmitoyl Transferase-1 (CPT-1) by inducing the expression of Peroxisome Proliferator-Activated Receptor alpha (PPAR-α)[72]. Therefore, it induces a decrease in circulating lipid levels, exerting a lipid-lowering role[71].

Adiponectin stimulates the membrane translocation of GLUT4 in adipocytes and muscle cells, promoting glucose consumption, and decrease glycogenolysis and gluconeogenesis in liver cells following the reduced expression of Glucose-6-Phosphatase (G6Pase) and PhosphoEnolPyruvate CarboxyKinase (PEPCK), which play a key role in these molecular pathways[73].

Several studies have reported that adiponectin exerts anti-atherogenic actions through different mechanisms. Firstly, it acts on the damaged vascular wall by reducing the ability of macrophages to transform into foamy cells[74]. Secondly, adiponectin inhibits sub-endothelial cholesterol accumulation, preventing atherosclerosis and plaque formation[71]. Finally, enhancing nitric acid production, this adipokine stimulates vasodilatation and increased blood flow[75]. Therefore, a cardioprotective effect of adiponectin follows. In particular, many reports proposed adiponectin as protective factor from thrombotic events and ischemic damage to the myocardium[76,77]. Moreover, epidemiological studies revealed an inverse correlation between circulating levels of adiponectin and risk of myocardial

infarction. Indeed, a low concentration of adiponectin may be associated with an increased risk of developing cardiovascular disease[78].

In contrast to most other adipokines, adiponectin is well recognized as an anti-inflammatory agent. Particularly, *in vitro* studies revealed its capability to inhibit the Nuclear Factor kappa light-chain-enhancer of activated B cells (NF-kB) pathway in monocyte-derived cell types in order to enhance the expression levels of anti-inflammatory cytokines interleukin 10 (IL-10), IL-6, TNF-α, and Interferon-gamma (IFNγ)[79,80]. Thus, low adiponectin levels cause increased expression of pro-inflammatory cytokines resulting in chronic inflammation and inflammation-associated cancers.

Moreover, many evidence suggests adiponectin as a crucial modulator of reproductive events, with positive effects on ovulation, implantation, and fetal growth and development[81]. Finally, although mechanism of action is still being studied, adiponectin plays an important role in bone homeostasis[82].

Thus, based on the function it exerts, adiponectin has been proposed as a protective factor against the development of obesity-related disorders, such as metabolic syndrome, diabetes, cardiovascular diseases and malignancies, including breast cancer [31].

2.3 Adiponectin signaling pathway

The binding of adiponectin to its receptor activates various signaling molecules responsible for the different physiological effects of this adipokine (Figure 7).

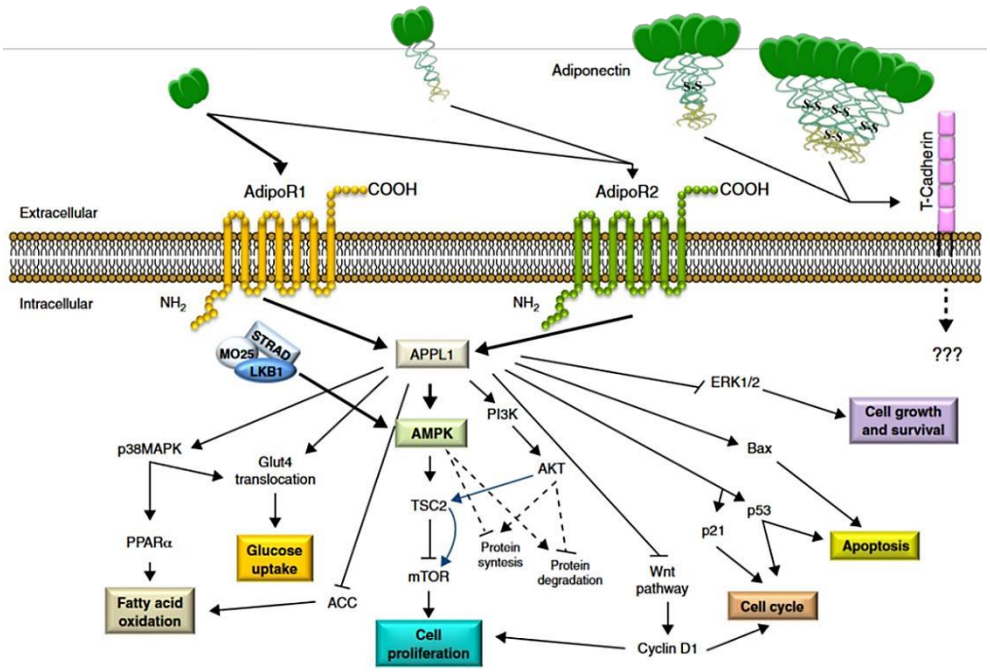


Figure 7. Adiponectin signaling pathway

The signal transduction is mainly regulated by the adaptor protein APPL1, which consists of three functional domains: a NH2-terminal leucine zipper motif (BAR domain), followed by a pleckstrin homology (PH) domain, and a phospho-tyrosine binding (PTB) domain near the COOH terminus[83]. AdipoR1 and AdipoR2 interact with the PTB domain of APPL-1 through their N-terminal intracellular region, thereby inducing adiponectin actions through the sequential activation of downstream signaling. In particular, Adiponectin exerts its effects through the activation of AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), PI3K/Akt, MAPK, PPAR-α, signal transducer and activator of transcription (STAT3) and NF-kB[36]. However, adiponectin is involved in AMPK activation not only through APPL-1 action but also through increased Adenosine monophosphate (AMP) levels and different cellular mediators encompassing calcium-dependent kinases and the liver kinase B1 (LKB1)[84].

Upon adiponectin binding to its receptor, APPL-1 binds and activates Protein Phosphatase 2A (PP2A), causing dephosphorylation and inactivation of Protein Kinase C (PKC). This, in turn, is responsible for the dephosphorylation of LKB1 at its Ser307 and the activation of AMPK[85]. AMPK is a sensor of cellular energy charge, which regulates physiological processes that consume or regenerate adenosine triphosphate (ATP) to restore the energy homeostasis in the cell. Specifically, activation of AMPK, which occurs during a condition of ATP depletion and increased adenosine diphosphate (ADP) and AMP levels, restores cellular energy homeostasis promoting glucose uptake and utilization, which results in an increased fatty-acid oxidation, and reducing energy-consuming anabolic processes, such as protein synthesis and gluconeogenesis in the liver[86]. One of the downstream proteins of AMPK is represented by mTOR. In particular, active AMPK inhibits mTOR signaling through a molecular mechanism that involves the phosphorylation of Tuberous Sclerosis Protein 2 (TSC2) at Thr1387. This phosphorylation enhances the activity of Tuberous Sclerosis Protein 1 (TSC1)/TSC2 complex to suppress mTOR signaling and its downstream effectors ribosomal p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E (eIF4E)-Binding Protein 1 (4E-BP1), via TSC2 GTPase-activating protein (GAP) activity[87].

APPL-1 also promotes the activation of insulin receptor substrate proteins, which act as docking platforms for the p85 regulatory subunit of the PI3K, inducing the formation of phosphatidylinositol 3,4,5-triphosphate (PIP3). The activation of PI3K/Akt signaling leads to a cascade of events resulting in cellular survival, growth and proliferation, and an increase in glycolysis and fatty acid synthesis. In

addition, Akt can be considered another key regulator of the TSC2 pathway due to its ability to remove the inhibitory effect of AMPK/TSC2 axis on Ras homolog Enriched in Brain (Rheb), through the directly phosphorylation of TCS2 at Ser939 and Thr1462. These molecular events affect the activation of both mTOR and its downstream protein p70S6k, with the consequent maintenance of cell growth and survival.

The proliferative effects of adiponectin can also be explained by the activation, through APPL-1 mediation, of MAPKs superfamily members, including c-Jun N-terminal Kinases (JNK), MAPKp38 and extracellular signal-regulated kinases (ERK) 1/2. In particular, ERK1/2-MAPK signaling has a pivotal role in cell cycle initiation, cell growth, and survival[88], as well as in ceramide catabolism and biosynthesis of the anti-apoptotic molecule S1P, involved in intracellular calcium production and AMPK activation. JNK and MAPKp38, instead, show variable effects on cell proliferation and apoptosis depending on cellular context. Overall, these actions support cell survival, nutrient uptake and utilization, and mitochondrial proliferation[36,89].

Noteworthy, adiponectin antagonizes leptin-induced STAT3 signaling, up-regulating the Suppressor Of Cytokine Signaling 2 (SOCS2). Additionally, many reports have pointed the adiponectin-mediated up-regulation of Protein Tyrosine phosphatase 1B (PT1B) expression, which induce a decrease in STAT3 phosphorylation, as another crucial mechanism through which this adipokine hamper leptin signaling[90].

Furthermore, adiponectin, through binding to AdipoR2, drastically enhances the expression levels and the activity of PPARα, a transcription factor responsible for

the up-regulation of ACO and UCPs. Specifically, PPAR-α stimulation increases fatty-acid combustion and energy consumption resulting in reduced triglycerides content in the liver and skeletal muscle and improved of insulin sensitivity *in vivo*[68]. Anti-inflammatory and anti-atherosclerotic properties of adiponectin also appear to depend on its ability to suppress the inhibitor of NF-kB phosphorylation. Indeed, the inhibition of NF-kB pathway could represent an important molecular mechanism for monocyte adhesion inhibition to endothelial cells[91].

Instead, adiponectin exerts anti-migratory effects stimulating the inhibition of Wnt/β-catenin signaling pathway, important for cancer progression[92]. It is well known that activation of the cell surface receptor, frizzled, by Wnt, leads to inactivation of glycogen synthase kinase-3β (GSK3β) and accumulation of β-catenin in the nucleus, where it is involved in the regulation of gene transcription[93]. Finally, adiponectin influences cell cycle progression modulating the expression levels of several proteins. In particular, it stimulates cell-cycle arrest and apoptosis through the down-regulation of c-myc, cyclin D1, and B cell lymphoma 2 (Bcl-2) and the up-regulation of p53, p21 and Bcl-2 Associated X-protein (Bax)[94].

2.4 Adiponectin and breast cancer

Many studies have evaluated the impact of adipokines in breast cancer progression and recurrence, suggesting a crucial role of adiponectin in obesity-related breast cancer. Indeed, it has been well recognized that the pathogenesis of mammary tumor is not only linked to genetic alterations but also widely correlated to interactions between malignant cells and components of the breast microenvironment, which can influence the phenotype of the neoplastic cells and

tumor progression. In particular, within the mammary gland, epithelial cells included in peri-glandular adipose tissue are exposed to both circulating and locally secreted adipokines from adjacent adipocytes. Therefore, the paracrine actions, as well as the endocrine effects of the hormones can affect breast cancer development[95,96].

Several reports highlighted that hypoadiponectinemia correlates with increased breast cancer risk and contributes to a more aggressive tumor phenotype[63,64,97]. This inverse correlation appears to be stronger in postmenopausal women since the ability of adiponectin to affect breast cancer cell proliferation in a low estrogen environment[97-99]. In contrast to these findings, different meta-analysis have demonstrated an increased incidence of intraepithelial breast tumor development and a higher risk of invasiveness in pre-menopausal women with baseline adiponectin levels[36]. In particular, low concentrations of adiponectin stimulate a greater production of pro-inflammatory cytokines, insulin and IGF-1 and support protein and fatty acid synthesis resulting in tumor growth and progression, regardless of age[100]. In addition, many evidences displayed that breast tumors with a higher histological grade and a greater metastatic capability are usually associated with low plasma adiponectin levels[97,99,101].

Actually, the role of this adipokine on breast tumorigenesis is still unclear. Indeed, many authors have proposed adiponectin as a protective factor against breast cancer due to its ability to activate various signaling pathways that prevent malignant transformation[102]. Specifically, adiponectin negatively regulates angiogenesis by inhibiting endothelial cell proliferation and, through the activation of caspases 3, 8 and 9, stimulates a cascade of molecular events that results in apoptosis[103].

However, the controversial role of adiponectin can be explained by the action of the various adiponectin isoforms, characterized by different molecular weights. Particularly, reduced levels of the HMW isoform seems to be related to an increased risk of developing breast cancer[104]. Moreover, adiponectin involvement in breast tumorigenesis is strongly dependent on the cell types[44]. In fact, *in vitro* and *in vivo* studies highlighted a dichotomic effect on breast tumor growth and progression in relationship to ERα expression[105–107].

2.4.1 Adiponectin and ERα-Negative Breast Cancer

Adiponectin is known to inhibit the growth of ERα-negative breast cancer cells mediating the activation of signal molecules able to switch off specific proliferative signaling pathways[106,108-110]. In particular, many studies have demonstrated that this adipokine stimulates phosphorylation events that induce the activation of AMPK; once activated, AMPK promotes the formation of TSC1/TSC2 complex, resulting in inhibition of mTOR signaling, thus interfering with breast cancer cell growth[107]. However, it is well documented that adiponectin enhances anti-proliferative response in ERα-negative breast cancer cells also regulating the expression levels of genes involved in apoptosis and cell cycle. Indeed, several *in vitro* and *in vivo* studies have revealed that low-adiponectin concentrations support in ERα-negative MDA-MB-231 breast cancer cells the up-regulation of pro-apoptotic factors and negative regulators of cell cycle, such as Bax, p21, and p53, as well as the down-regulation of Cyclin D1. Overall, these events contribute to cell cycle arrest at G0/G1 phase[105]. In addition, a RNA sequencing (RNA-seq) analysis performed in adiponectin-treated MDA-MB-231 cells showed a down-regulation of cyclin A2

and cyclin-dependent kinase 1, implicated in G1/S and G2/M transition of cell cycle, concomitant to a greater expression of cell cycle inhibitors such as TGFBR1, IGFBP3, and MAGED1[107,111,112]. Moreover, the analysis revealed that adiponectin counteracts cell growth also through an increased expression of FAS, BIK, BID, and caspases, important for apoptosis activation[107,113], and a reduced expression of CKAP2 and RRM1, responsible of microtubule assembly and DNA replication during S phase[105,114]. Further investigations demonstrated that low doses of adiponectin inhibited ERK1/2 signaling[94] and limited the anchorage-independent growth[94,105] and lamellopodia formation of MDA-MB-231 breast cancer cells, preventing cell migration and invasion[115,116].

2.4.2 Adiponectin and ERα-Positive Breast Cancer

Many authors have elucidated the complex cross talk existing between adiponectin and ER signaling in breast cancer cells. Evidence that full-length adiponectin stimulates the E2-induced proliferation rate in ERα-positive MCF-7 breast cancer cells[117] has prompted further investigations to clarify the molecular mechanisms through which this adipokine can act as stimulatory factor.

In particular, Mauro et al. demonstrated that low adiponectin doses (1 and 5 μg/ml), which mimic obesity condition, exert proliferative actions in ERα-positive breast cancer cells, which are markedly abrogated in the presence of ERα pharmacological inhibition (ICI 182,780) or ERα knocking down. Mechanistically, it has displayed that globular adiponectin induces the interaction between its receptor Adipo R1 and a complex of protein including APPL1, ERα, IGF-IR, and c-Src, leading to MAPK phosphorylation. This contributes to trans-activate ERα at genomic level through

the phosphorylation at Ser118. The ability of this adipokine to activate ERα is also confirmed by the increased expression of estrogen-responsive genes, such as Catepsin D and pS2[106]. These findings were corroborated by *in vivo* studies that demonstrated how the injection of adiponectin-treated MCF-7 cells into nude mice stimulated breast tumor growth. Furthermore, the proliferative effects of adiponectin in relation to ERα status were also confirmed by an increased expression of Cyclin D1, in terms of mRNA and protein content, in adiponectin-treated ERα-positive cells[105].

Further studies revealed that low adiponectin levels counteract the onco-suppressor role of LKB1 in ERα-positive breast cancer cells. In particular, adiponectin exposure stimulates ERα /LKB1 interaction both in the cytosol and in the nucleus, preventing LKB1 association with the 2-scaffold protein STRAD and MO25 required to activate AMPK signaling. Consistent with these findings, LKB1 has been proposed as ERα coactivator. Indeed, adiponectin, promoting ERα/LKB1 interaction, induces nuclear LKB1 localization and stimulates its recruitment to an ERE sequence in the promoter region of pS2, an estrogen-dependent gene[107].

However, the adiponectin-mediated transactivation of ERα/LKB1 complex is also responsible for increased expression levels of E-cadherin and LKB1 itself. Specifically, it has been demonstrated that adiponectin signaling promotes in MCF-7 cells an increased recruitment of ERα and LKB1 on Sp1 responsive element present on E-cadherin promoter, as well as an enhanced binding of RNA polymerase II, resulting in activation of E-cadherin gene promoter. In addition, in ERα-positive breast cancer cells adiponectin exposure increases ERα recruitment on the ERE binding sequence to the LKB1 regulatory region, concomitantly with a

high association of pCAF and RNA Polymerase II, suggesting that adiponectin is able to activate the LKB1 transcriptional machinery. All these events were abrogated in the presence of the ERα or LKB1 specific siRNAs[118].

Since E-cadherin links cell polarity and growth, it has been evaluated if the adiponectin-enhanced E-cadherin expression could affect the localization of proteins cooperating in cell polarity, such as LKB1 and Cdc42. In particular, Naimo et al demonstrated that, in adiponectin-treated ERα-negative breast cancer cells LKB1 and Cdc42 co-localize with E-cadherin in the cytosol cooperating in the maintenance of cell polarity, while in MCF-7 cells adiponectin induces the loss of the cytosolic cooperation of E-cadherin with LKB1 and Cdc42, due to their nuclear localization. Consistent with these data, adiponectin negatively interferes with mammary epithelial cell polarity and results in not oriented cell motility. Thus, experimental evidence revealed that E-cadherin, in adiponectin-treated ERα-positive breast cancer cells, is mainly involved in stimulating cell proliferation rather than cell polarity maintenance. These data were also confirmed by *in vivo* studies. In particular, the orthotopic implantation of adiponectin-treated MCF-7 cells revealed the ability of this adipokine to increase E-cadherin-mediated breast cancer growth. Moreover, it is worth noting that experimental metastasis assay showed that mice tail vein injection of adiponectin-treated MCF-7 cells is responsible of a higher distant metastatic burden[118].

3. Breast cancer stem cells

3.1 Discovery and origin

Emerging evidence refutes the theory that the main cause of cancer initiation and maintenance arises from the accumulation of genetic alterations in somatic cells, which drive the transformation of normal human cells into highly malignant derivatives[119]. Indeed, tumor mass is described as a heterogeneous population of cells in which it is possible to highlight a small subpopulation of undifferentiated cells, named as Cancer Stem Cells (CSCs) or cancer-initiating cells, able to originate the differentiated cells that comprise the bulk of the tumor and characterized by a high tumorigenic potential[120]. Particularly, CSCs hypothesis suggests that the strong self-renewal ability and the multi-lineage differentiation capacity of CSCs confer them a crucial role in tumorigenesis and cancer progression. Normally, CSCs undergo asymmetric division that allows them to originate another CSC, with unlimited proliferative potential, and a cancer cell characterized by limited lifespan and capability to integrate with the tumor mass by exploiting differentiation mechanisms[119]. However, CSCs can also divide symmetrically, usually as a response to stress condition, generating two daughter cells with stem-like phenotype, in order to maintain CSCs progeny and excessively increase tumor growth and repopulation[121].

Although the fraction of CSCs expressed in solid tumors represents a very small percentage of all tumor cells (approximately 2%), they exert a primary role in establishing intratumoral heterogeneity[122]. Moreover, it is worth noting that the

identification of CSCs assumes clinical relevance since it has prognostic and therapeutic implications.

The isolation of leukemic stem cells from the mononuclear cells of the blood of human acute myeloid leukemia was the first evidence supporting CSCs existence. It has been demonstrated that these cells, which exhibit an exclusive $CD34^{+}CD38^{-}$ phenotype, share similar features with Normal Stem Cells (NSCs) and can be differentiated into leukemic blasts[123]. Since the concept of CSC was introduced, scientists have attempted to isolate CSCs from other types of cancer. In particular, breast cancer stem cells (BCSCs) were firstly described by All-Hajj et al. in 2003. The study revealed that injection into the mammary fat pad of non-obese diabetic severe combined immunodeficient (NOD/SCID) mice of approximately a hundred cells isolated from primary breast tumors and metastatic pleural effusions and expressing the adhesion molecule CD44, the epithelial surface antigen (ESA) but with CD24 (a ligand for P-selectin) absente or poorly expressed, was able to originate tumors. In contrast, injection of much greater numbers of breast cancer cells with $CD44^{-}/CD24^{+}$ phenotype, obtained from the same samples, failed to induce tumorigenesis. Consistent with these findings, further *in vitro* studies demonstrated that $CD44^{+}CD24^{-}$ cells, identified as BCSCs, showed self-renewal, mammospheres formation, and chemotherapy resistance abilities, as well as capability to differentiate into cells of tumor bulk[124].

Although the investigation regarding the origin of BCSCs has attracted much controversy among scientists, the clonal evolution theory and the cancer stem cell theory seems to be the two most popular theories explaining how CSCs originate within the tumor mass.

According to clonal evolution model, proposed by Peter Nowell in 1976[125], individual cancer cells can be affected over time by stochastic genetic/epigenetic changes, which lead to the selection of the fittest and most aggressive clones[126]. Hence, these changes predict the existence of genetic heterogeneity among cancer cells resulting from a competition process, which will drive the natural selection and the evolution of BCSCs with better survival fitness. During tumor progression, genetic instability represents the main cause of the production of cells with additional mutations and new features, which can confer them a growth advantage over other tumor cells, such as resistance to cell death programs. Therefore, these events may orchestrate the appearance of new cellular variant resistant to therapy and with invasive and metastatic property[125,127]. In contrast to clonal evolution theory, CSC model, identified as hierarchical approach, states that only cancer cells with stem-like features are involved in tumor initiation, progression, and recurrence due to their high tumorigenic potential and their ability to self-renew indefinitely (Figure 8).

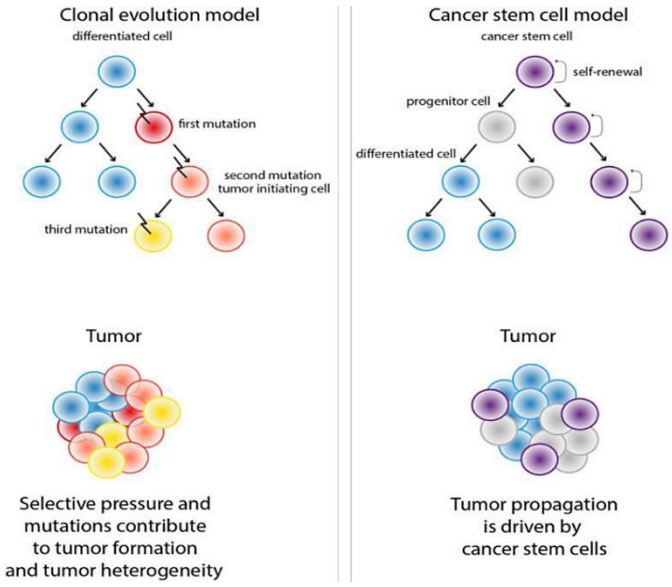


Figure 8. Clonal evolution model vs Cancer stem cell model

Through both symmetrical and asymmetrical cell division, CSCs can maintain a stem-like cells pool and can contribute to intratumoral heterogeneity by supporting differentiation programs capable of giving rise to a wide range of different cell types within a tumor. However, it is important to note that the differentiation program is not only one-way route but it could be reversible. Indeed, differentiated cancer cells can undergo a process of phenotype reversal, leading to the acquisition of CSC-like characteristics through a dedifferentiation program. Moreover, many authors have reported the existence of various subpopulations of CSCs with different biochemical, biophysical, and metabolic signatures within a tumor, responsible of drug resistance and tumor heterogeneity[128].

3.2 BCSCs biomarkers

CSCs are phenotypically different from the other cells existing within the tumor bulk and there are no universal markers for the specific recognition of this subpopulation. However, BCSCs are generally identified and sorted by high CD44/CD24 ratio and increased aldehyde dehydrogenase 1 (ALDH1) activity[120].

CD44 is a transmembrane glycoprotein receptor that bind hyaluronic acid (HA), important component of extracellular matrix (ECM). Through the interaction with HA, CD44 regulates several molecular mechanisms, including cell adhesion to ECM components, tyrosine phosphorylation of cytoskeletal proteins and activation of RhoA/RhoC, Rac1 and Cdc42[129]. Overall, these molecular events lead to actin cytoskeletal remodelling, actin filament assembly, tumor cell migration and invasion[130].

CD24, adhesion molecule binding P-selectin, is involved in cellular adhesion, proliferation and metastasis[131]. In particular, CD24 expression levels are very low or absent in BCSCs and *in vitro* studies evidenced that the up-regulation of CD24 counteracts stemness[132].

ALDH1 is a member of the aldehyde dehydrogenase family of proteins, which is critically responsible for the oxidation of retinol to retinoic acid, required for early differentiation of stem cells. ALDH1 is a marker of both human normal mammary stem cells and malignant mammary stem-like cells, and high ALDH1 activity is considered an independent predictor of poor clinical outcome and survival of breast cancer patients[133,134].

It is worth mentioning that, although $ALDH1^+$ cells have also been identified as tumor initiating cells, they partially overlap the isolated $CD44^+CD24^-$ population[135]. Specifically, many evidence suggest that BCSCs can undergo a phenotypic interconversion between epithelial and mesenchymal states and that each state is associated with different localisation within the tumor[136]. Indeed, while epithelial-like BCSCs, which express ALDH1, have a high proliferative index and are located in the central core of the tumor, $CD44^+CD24^-$ mesenchymal-like BCSCs are more quiescent and detected on the invasive front of the tumor. The mesenchymal state is essential in the first step of metastatization in order to promote cell dissemination in the circulation and to generate micrometastasis. However, when the mesenchymal cells of invasive edge constitute a new tumor mass becoming the central area of the tumor, it is possible to observe the switch to the $ALDH1^+$ epithelial state, crucial to macrometastasis formation. Moreover, several studies showed the existence of a third subpopulation of BCSCs with

$CD44^{+}CD24^{-}ALDH1^{+}$ phenotype, identified as the BCSCs population with the highest tumorigenic potential and invasion capability[136].

However, additional markers have been reported to identify BCSCs populations, including several transcription factors and various cell surface markers.

The Octamer-binding transcription factor-4 (Oct-4), an embryonic stem cell marker, play a crucial role in stem cell self-renewal. Notably, comparison between the $Oct\text{-}4^{high}$ cell population and the $Oct\text{-}4^{low}$ cell population in the 4T1 mouse breast cancer model showed that $Oct\text{-}4^{high}$ 4T1 cells have a greater ability of mammospheres formation *in vitro* and increased levels of stem cell markers[137]. Consistent with these data, the knockdown of Oct-4 in MCF-7 cells induces apoptosis, reduces BCSCs features, and inhibits tumor growth[138].

Sox2 is a transcription factor necessary for tumorspheres formation and experimental evidence showed that breast cancer cells Sox2 silencing caused a delay in tumor formation in mice xenografts[139]. Moreover, TRPS1 and FOXO3a - mediated Sox2 inhibition reduces BCSCs tumorigenicity[140,141].

Further studies demonstrated that Sox9 knockdown significantly inhibits the tumorigenicity of MDA-MB-231 cells, with a 70-fold decrease in tumor initiation capacity and a 40-fold increase in the ability to inhibit lung metastasis compared to the control. Interestingly, it has also been highlighted Sox9 and SLUG involvement in the synergistic reversal of differentiated luminal cells into mammary stem cells[142].

Beyond CD44 and CD24, other cell surface proteins are commonly used as BCSCs biomarker, including integrin subunit α6, Epithelial cell adhesion molecule (EpCAM), C-X-C motif chemokine receptor 4 (CXCR4) and CD133.

Integrin subunit α6, also known as CD49f, is a glycoprotein that acts as receptor for several laminins and plays a key role in cell adhesion to ECM[143]. α6B variant represents the isoform commonly associated with a CSC phenotype[144].

EpCAM, overexpressed in BCSCs, is a transmembrane glycoprotein involved in several biological processes and it is recognized as a well-established CSC marker[145] as it has the ability to promote BCSCs survival through the activation of the Wnt signaling pathway[146]. Furthermore, high expression levels of EpCAM are detected in circulating tumor cells (CTCs), which seem to exhibit CSC functions and activity, allowing the survival of cancer cells in anoikis-independent conditions[147,148].

CXCR4 is considered a prognostic marker of BCSCs. In particular, CXCR4 and its ligand, CXCL12 (SDF-1), may regulate breast cancer cells dissemination from the primary site, trans-endothelial migration, and BCSCs trafficking. In fact, the chemoattractant CXCL12 provides directional guidance to $CXCR4^+$ BCSCs toward the secondary metastatic site, promoting metastasis formation[149,150]. It is worth noting that a gene expression profile study evidenced that CXCR1 was up-regulated in $ALDH^+$ BCSCs of different breast cancer cell lines[151].

CD133 is identified as a potential BCSCs biomarker since its important role in suppressing the differentiation processes. Wright et al. demonstrated that $CD133^+$ cells derived from BRCA1 murine breast tumors exhibit enhanced colony formation efficiency, increased proliferative rate and high tumorigenicity[152]. Furthermore, additional studies revealed that $ALDH^+/CD44^+/CD133^+$ cell lines, derived from invasive breast cancer, are characterized by increased migration and invasion capacity and greater metastatic potential *in vitro* and *in vivo*[134].

3.3 Signaling pathways regulating BCSCs functions

CSCs phenotype is influenced by intricate signaling pathways that, cooperating with each other, are able to modulate self-renewal, differentiation, proliferation and survival of CSCs. It is well documented as in mammary gland, the generation and the maintenance of BCSCs can result from deregulation of several pathway, encompassing Notch, Hedgehog (Hh), Wnt, Hippo, NF-kB, Transforming Growth Factor-beta (TGF-β) pathways (Figure 9).

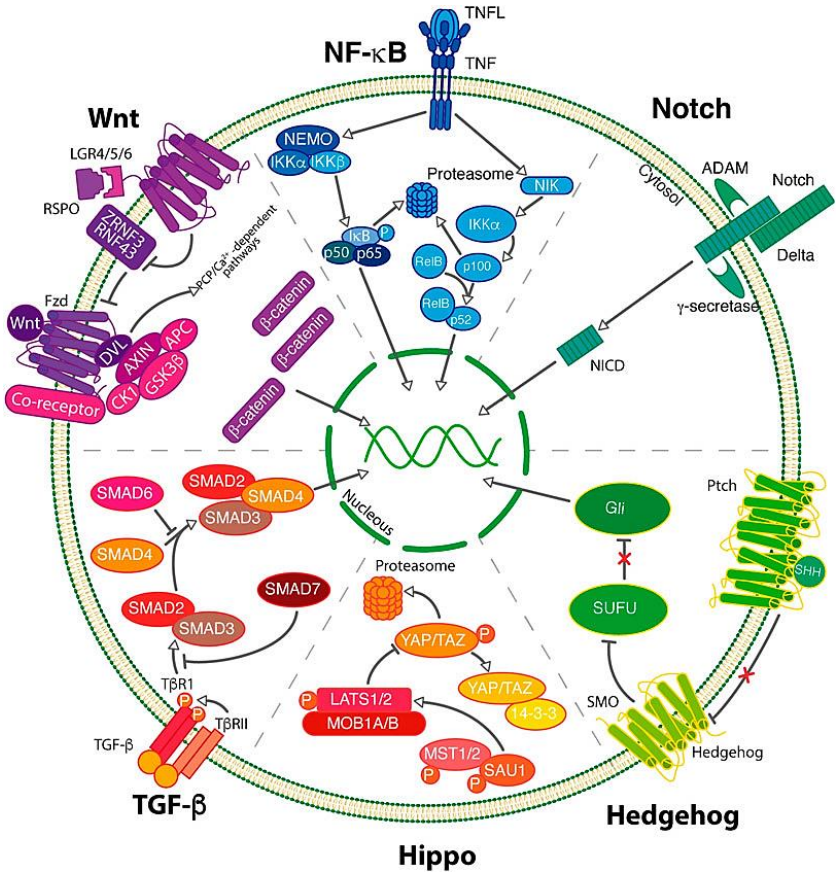


Figure 9. Signaling pathway regulating BCSCs

The signaling molecules and components of these pathways, which are repressed or abnormally activated in BCSCs, have been extensively investigated as therapeutic target to eradicate BCSCs[153].

The Notch signaling pathway is commonly activated in both normal and malignant stem cells. While activation of Notch signaling in normal stem cells allows them to maintain a balance between self-renewal and differentiation, abnormal activation of Notch signaling in BCSCs promotes self-renewal, induces epithelial mesenchymal transition (EMT) and supports the metastatic process[154]. It has been described four NOTCH receptors (NOTCH1, 2, 3, and 4) and five ligands (Delta-like-1 or DLL-1, 3, 4 and Jagged1 and 2).

The ligand binding to its receptor induces the proteolytic cleavage of the NOTCH intracellular domain (NICD), which subsequently translocates to the nucleus and interacts with the transcription complex to activate downstream events, such as the expression of HES/HEY family of genes involved in tissue-specific transcription factors repression[155]. Since the Notch pathway mainly targets genes that sustain proliferation and inhibit apoptosis, aberrant activation of this pathway in breast cancer results in uncontrolled development and maintenance of BCSCs. Consistent with these data, it has been evidenced that the use of antibodies specifically towards Notch receptors lead to inhibition of tumor growth[156]. Similarly, experimental studies revealed that the knockdown of Notch-1 exerts a key role in tumor regression in a mouse mammary tumor model[157]. Additionally, accumulating evidence showed that the inhibition of Notch signaling sensitises BCSCs to chemotherapy and radiation[158].

An abnormal up-regulation of Hh pathway was observed in several CSCs model, including breast cancer[159]. The aberrant activation of Hh pathway in BCSCs stimulates increased expression levels of Sonic hedgehog-(Shh), one of the ligands in the Hh pathway, and GLI, transcription factor involved in breast cancer

development and progression through angiogenesis activation[160]. Specifically, Hh signaling can support self-renewal ability and metastasis of CSCs modulating the expression of several marker that identify a stem-like phenotype, including ALDH1, Oct-4, SNAIL, TWIST1, C-Myc, Jagged1, Nanog. Currently, several Hh signaling pathway inhibitors have been developed targeting specifically smoothened (SMO), a transmembrane signaling protein that mediates Hh downstream signaling pathway. Indeed, it has been showed that inhibition of Hh signaling, using SMO antagonist, makes BCSCs responsive to chemotherapy[161].

Increased activation of both canonical and non-canonical Wnt signaling is responsible of BCSCs self-renewal, bulk-tumor expansion and therapy resistance. While the canonical Wnt/β-catenin pathway influences stem cells self-renewal and progenitor cells proliferation or differentiation, the non-canonical Wnt signaling cascades contributes mainly to suppression of the canonical Wnt pathway and BCSCs survival[162]. Moreover, both canonical and non-canonical Wnt signaling pathway play a key role in the regulation of cell invasion and migration, as well as metastasis. Consistent with these results, the expression levels of Wnt signaling pathway-related proteins are limited in normal breast stem cells, but increased in BCSCs. Furthermore, several reports have been showed to support the idea that blocking canonical Wnt/β-catenin signaling significantly reduces the number of BCSCs in breast cancer. In particular, the inhibition of Wnt cascade can derive by antagonizing receptors that initiate the Wnt pathway, blocking the proteins involved in cell signal transduction or hampering the interactions of transcription initiation factors complexes[163].

It is evident that TAZ, an effector of the Hippo pathway, is closely associated to the proliferation, migration and transformation ability of BCSCs. Specifically, TAZ activation, promoted by vascular endothelial growth factor (VEGF)/neuropilin-2 (NRP2) signaling through a Rac1-dependent mechanism, increases the ability to mammospheres formation. However, it has been reported that also Tumor Necrosis Factor-alpha (TNF-α) can stimulate TAZ expression levels[164].

The NF-κB signaling pathway is overexpressed in BCSCs and regulates their self-renewal and differentiation[165]. The NF-kB cascade include five related transcription factors (p50, p52, RelA, c-Rel, and RelB) and its activation results in ubiquitin ligase-dependent degradation of IκB and nuclear translocation of NF-κB protein complexes. It is worth noting that NF-κB signaling, stimulating the expression of inflammatory cytokines, including IL-6 and IL-8, and inducing EMT, sustain the maintenance of BCSCs[166]. NFκB cascade is activated by the canonical and the non-canonical pathway, both involved in the chemoresistance, tumorigenesis, and self-renewal of BCSCs[166-169]. The canonical signaling is rapidly inducible and activated by different pro-inflammatory cytokines including TNF-α. In particular, TNF-α increases the proportion of BCSCs in human breast cancer cell lines by up-regulating TAZ expression[170]. An abnormal activation of canonical NF-κB signaling is responsible of chemotherapy resistance in quiescent BCSCs[167]. In contrast to canonical signaling, the activation of non-canonical pathway is slow and persistent and acts enhancing the expression of stem cell markers, regulating self-renewal ability, and promoting tumorigenesis and invasiveness of CSCs through IKKα[171].

It is well documented that TGF-β is an important regulator of BCSCs by inducing EMT and regulating apoptosis and drug resistance[171,172]. In particular, TGF-β phosphorylation at ser 69 and 74 recruits SMAD3/p53 complex and regulates the transcription of BCSCs resistance genes[173]. Moreover, it has been demonstrated that TGF-β2 expression correlates with the BCSCs marker ALDH1, representing a bad prognosis index. Finally, TGF-β signaling also increases the metastatic capacity of $CD44^{+}CD24^{-}$ BCSCs and enhances the expression levels of BCSC markers, including Nanog, Pou5f1, and Sox2[172].

3.4 BCSCs and drug-resistance

Although conventional therapies target proliferating cells and are commonly successful in reducing the size of primary tumors, the complete tumor eradication is often difficult to achieve due to several factors, including the existence of BCSCs. Indeed, this stem-like subpopulation can escape the standard therapeutic approach through adaptation to several strategies[120] (Figure 10).

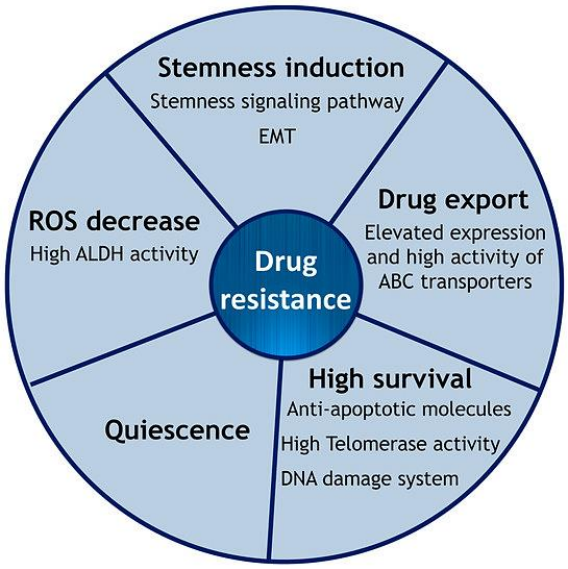


Figure 10. Hallmarks of BCSCs drug resistance

One of the most important features of BCSCs is the ability to maintain themselves in the G0 phase of cell cycle, thereby remaining in a quiescent state. The dormancy allows them to evade systemic therapies, obtaining protection from chemotherapy and/or radiation damage[174]. Related to this, several studies indicate that treatment with chemotherapy or radiotherapy can modify divisional dynamics of BCSCs, favoring symmetrical division that lead to an enrichment of $CD44^{+}/CD24^{-/low}$ cells with greater chemo/radio-resistance[175]. Thus, the development of drug resistance confers to BCSCs a selective advantage over the non-CSCs, which supports the "survival of the fittest" hypothesis. However, it is worth noting that they preserve the ability to leave quiescence state and re-enter the cell cycle in response to mitotic stimulation[176]. In addition to dormancy, BCSCs exploit several mechanisms to escape to death signals, including imbalance between pro-apoptotic and anti-apoptotic proteins, down-regulation of death receptors and increased expression of inhibitors of apoptosis family proteins. However, while programmed cell death is hampered, autophagy is induced in BCSCs since it provides them metabolic flexibility indispensable to survive in oxygen- or nutrient-poor conditions[177]. Increasing evidence suggest that this self-degradation process plays a crucial role in BCSCs dormancy, stemness and maintenance[178]. Moreover, promoting an enrichment of BCSCs, autophagy contributes to the development of drug resistance[179].

The resistance of BCSCs to radiation and cytotoxic agents may be also depend on the presence of an efficient DNA damage response (DDR) machinery, which contributes to counteract apoptosis and to up-regulate DNA damage checkpoints genes. Indeed, BCSCs exhibit a constitutive activation of Ataxia Telangiectasia

Mutated (ATM) protein kinase, key sensor of DNA damage, and increased activity of Checkpoint Kinase 1 (CHK1) and Checkpoint Kinase 2 (CHK2), master regulators of checkpoint-mediated cell cycle arrest and activation of DNA repair in response to damage[180,181].

Notably, ionizing radiation used during cancer therapy induces DNA damage through the production of intracellular Reactive Oxygen Species (ROS) able to interact with DNA, as well as with proteins and lipids. However, in BCSCs the increase of intracellular ROS levels triggers the overexpression of radical scavenger capable of reducing ROS oxidation and maintaining redox balance[120]. It has been reported that nrf2, a transcription factor that mitigates oxidative stress by regulating the expression of genes involved in the cellular antioxidant response, is highly expressed in BCSCs to maintain a relatively low level of ROS and is considered a mediator of BCSCs resistance due to its ability to support drug efflux[182]. Specifically, BCSCs express high levels of ATP-binding cassette (ABC) transporters involved in chemo drugs extrusion, like anthracycline or taxanes, and thereby responsible for the acquisition of multi-drug resistance phenotype. Thus, the high expression of ABC transporters, such as ABCB1 (MDR1), ABCG2 (BCRP1), ABCC11 (MRP8), and ABCB, promotes the survival of BCSCs to chemotherapy[183]. Moreover, these cells are also referred as "side population or SP" due to the ability of excluding dyes such as Hoechst 33342 or Rhodamine.

ABC transporters not only contribute to establishing the drug resistance via increased efflux of chemo drugs but also stimulate EMT. In particular, Saxena et al, demonstrating that ABC transporter promoters display different binding sites for EMT-inducing transcription factors and overexpression of TWIST, SNAIL, and

FOXC2 enhances the promoter activity of ABC transporters, highlighted some of the molecular mechanisms that associate EMT with multi drug-resistance[184].

Moreover, EMT plasticity may play a crucial role in BCSC-mediated therapeutic resistance since it allows BCSCs to switch between a proliferative epithelial-like state and a quiescent mesenchymal-like state[185,186]. It has been evidenced that metastatic cancer cells, generated post-EMT, manifested mesenchymal-like phenotype and $CD44^{high}/CD24^{low}$ signature and were able to form mammospheres unlike their $CD44^{low}/CD24^{high}$ counterpart. Thus, an up-regulation of EMT-related genes may induce the emergence of dedifferentiated cells with CSC phenotype, leading to metastasis and drug resistance[187].

Interestingly, BCSCs are mainly detected in hypoxic areas and this characteristic make them more resistance to radiotherapy and chemotherapy. In particular, hypoxia induces an enrichment of BCSCs subpopulation supporting the expression levels of hypoxia-inducible factors, like HIF-1α and HIF-2α, critical regulators of cancer stemness. While HIF-1α sustains the up-regulation of survival genes in hypoxic conditions, HIF-2α interacts with the promoter of Nanog and Oct-4, thereby promoting the expression of stem-like markers and drug resistance. Experimental studies revealed that chemotherapy treatment of various human breast cancer cell lines is responsible of greater expression of HIF, which increase BCSCs population through IL-6 and IL-8 signaling and MDR1 overexpression[188].

Finally, it is worth mentioning that also ALDH contributes to drug resistance since its ability to metabolically inactivate chemotherapeutic agents[189] and to protect BCSCs from ROS-associated toxic effects[190]. Indeed, *in vitro* and *in vivo* studies evidenced that $ALDH^{high}CD44^{+}$ subpopulation shows higher metastatic ability and

resistance to conventional cancer therapy rather than $ALDH^{low}CD44^{-}$ subpopulation[191].

Materials and Methods

1.1 Cell culture

Human breast cancer epithelial cell line MCF-7 was purchased from American Type Culture Collection (ATCC) and stored and authenticated according to supplier's instructions. Cells were maintained in DMEM/F-12, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (Life Technologies). Tamoxifen resistant variant cell line (TR MCF-7) derived from MCF-7 cells chronically treated with tamoxifen (10^{-6} M 4-hydroxytamoxifen) (Merck). Cells were incubated with 5% CO_2 at 37°C. For experimental purposes, cells were synchronized in phenol red-free and serum-free media for 24h and then untreated (control, C) or treated with globular adiponectin 5 μg/ml (A5) (Prospect) in a phenol-red free medium containing 10% dextran charcoal-stripped FBS. All experiments were performed with mycoplasma-free cells.

1.2 Mammosphere assay

WT and TR MCF-7 monolayer cells, grown in regular media to reach 80-90% confluency, were enzymatically and manually disaggregated to obtain single-cell suspension. Single cells were plated in ultralow attachment plates at a density of 500 cells/cm^2 in phenol red-free and serum-free DMEM/F12 supplemented with B-27 (Invitrogen) and 20 ng/ml Epidermal Growth Factor (Merck). Cell lines were pre-treated in adherence with adiponectin 5 μg/ml for 48h prior to plating in mammosphere culture. After 7 days, mammospheres ≥50 μm diameter (primary generation mammospheres) were counted using a microscope (x40 magnification),

collected, enzymatically dissociated and plated at the same seeding density used in the primary generation to obtain secondary mammospheres. No additional treatments were added in secondary generation. Mammosphere-forming efficiency (MFE) was calculated by dividing the number of mammospheres formed by the number of single cells seeded per well and expressed as percentage. Mammosphere data are presented as percentage of mammosphere formation in adiponectin-treated samples, normalized to percentage of mammosphere formation in control samples. Mammosphere self-renewal was calculated by dividing the number of secondary mammospheres formed by the number of primary mammospheres formed.

1.3 Flow cytometric detection of CD24, CD44 and ALDH1 expression

Secondary generation mammospheres were digested using PBS containing 0.25% trypsin 0.02% EDTA (Life Technologies) to obtain single-cell suspension. To detect CD44 and CD24 expression, cells were washed in PBS with 2.5% BSA and stained with FITC anti-human CD44 and PE anti-human CD24 (BD Biosciences), according to the supplier's protocol. Instead, to detect ALDH1 expression, cells were incubated in ice-cold Methanol for 10' at -20°C. Subsequently, pellet was resuspended in Triton 0.1% and incubated for 15' at room temperature (RT). Finally, cells were washed in PBS with 2.5% BSA and stained with FITC anti-human ALDH1A1 antibody (Santa Cruz Biotechnology), according to the manufacturer's instructions. Flow cytometric analysis was performed with CytoFLEX flow cytometry (Beckman-Coulter) and acquisition data was obtained with CytExpert Beckman Coulter software.

1.4 Total RNA extraction, reverse transcription polymerase PCR and real-time RT-PCR assay

Total RNA was isolated using TRI-zol reagent (ThermoFisher Scientific) and reverse transcribed (2 μg) with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's instructions. cDNA was diluted 1:10 in nuclease-free water. Gene expression was evaluated by real-time reverse transcription PCR (qRT-PCR) with QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific) using SYBR Green Universal PCR Master Mix (Bio-Rad). Each sample was normalized on 18S mRNA content. Relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. The results were expressed as n-fold differences in gene expression relative to 18S rRNA and the calibrator, calculated using the ΔΔCT method as follows: n-fold = $2^{-(\Delta CT_{sample} - \Delta CT_{calibrator})}$, where the ΔCT values of the sample and calibrator were determined by subtracting the average CT value of the 18S rRNA reference gene from the average CT value of the different genes analyzed. Primers used are listed in Table 2.

Table 2. List of primers used

OLIGO NAME	SEQUENCE 5'-3'
ALPHA-SMA	Fw-AGACATCAGGGGGTGATGGT Rw-CATGGCTGGGACATTGAAAG
CD24	Fw-GCTCCTACCCACGCAGATT Rw-GTGAGACCACGAAGAGACTGG
CD44	Fw-GAAGAAGGTGTGGGCAGAAGA Rw-ACCATTTCCTGAGACTTGCTG
CYCLIN D1	Fw-GATGCCAACCTCCTCAACGAC Rw-CTCCTCGCACTTCTGTTCCTC
E-CADHERIN	Fw-TGCCCAGAAAATGAAAAAGG Rw-GTGTATGTGGCAATGCGTTC
NOTCH1	Fw-GTGACTGCTCCCTCAACTTCAAT Rw-CTGTCACAGTGGCCGTCACT
NOTCH2	Fw-CACCCCAGCTGCTACTCACA Rw-GCCAACCCAGCCTGCAT
NOTCH3	Fw-CCTGTCTTCCTGGGTTTGAG Rw-CAGAACTGGCCTGTGCACTC
NOTCH4	Fw-CCAACCCTGCGATAATGCGAG Rw-AGTCATCCGTTGAGACCCTGC
OCT-4	Fw-AGCGACTATGCACAACGAGA Rw-CCATAGCCTGGGTACCAAA
p21	Fw-GCATGACAGATTTCTACCACTCC Rw-AAGATGTAGAGCGGGCCTTT
SMAD4	Fw-GGAGCTCATCCTAGTAAATG Rw-GACGGGCATAGATCACATGA
SNAIL	Fw-CGAGTGGTTCTTCTGCGCTA Rw-GGGCTGCTGGAAGGTAAACT
SOX2	Fw-CACATGAAGGAGCACCCGGATTAT Rw-GTTCATGTGCGCGTAACTGTCCAT
SOX4	Fw-GGCCTCGAGCTGGGAATCGC Rw-GCCCACTCGGGGTCTTGCAC
SOX9	Fw-AACGCCGAGCTCAGCAAGA Rw-TTCTTGTGCTGCACGCGCA
TWIST	Fw-TCCAAATTCAAAGAAACAGGGCG Rw-CAGAATGCAGAGGTGTGAGGA
VIMENTIN	Fw-GAGAACTTTGCCGTTGAAGC Rw-GCTTCCTGTAGGTGGCAATC
Ki67	Fw-TCCTTTGGTGGGCACCTAAGACCTG Rw-TGATGGTTGAGGTCGTTCCTTGATG
18S	Fw-CGGCGACGACCCATTCGAAC Rw-GAATCGAACCCTGATTCCCCGTC

1.5 Cell cycle analysis

Disaggregated spheres were pelleted, washed with PBS and fixed in 50% methanol overnight (ON) at -20°C. Then, cells were stained with a solution containing 50 µg/ml propidium iodide (PI), 20 U/ml RNase and 0.1% Triton. Cell cycle phases were estimated as a percentage of a total of 10000 events. The DNA content was measured using CytoFLEX flow cytometry (Beckman-Coulter) and the data acquired using CytExpert Beckman Coulter software.

1.6 Western Blotting

Whole cell extracts were prepared 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, supplemented with protease and phosphatase inhibitors (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate) (Merck). The protein content was determined using Bradford dye reagent (Bio-Rad). Equal amounts of total protein were resolved, under denaturing conditions, on SDS-polyacrilamide gels and transferred onto a nitrocellulose membrane by electro-blotting. The membranes were blocked in 5% non-fat dry milk (Merck) and probed ON at 4°C with an appropriate dilution of specific primary antibodies. The antigen-antibody complex was detected by incubation of the membranes for 1h at RT with a peroxidase-coupled anti-IgG antibody and revealed using an enhanced chemiluminescence system (ECL, Santa Cruz Biotechnology). Primary antibody used are listed in Table 3.

Table 3. Catalog number and dilution of all the antibodies used

Antibody	Catalog number	Dilution	Manufacturer
Anti-BAD	sc-8044	1:500	Santa Cruz Biotechnology
Anti-BAX	bsm33279M	1:500	Bios, Massachusetts, USA
Anti-Bid/tBid	2003S	1:1000	Cell Signaling Technology
Anti-Bcl-2	sc-7382	1:500	Santa Cruz Biotechnology
Anti-Caspase 3	Sc-7272	1:500	Santa Cruz Biotechnology
Anti-Caspase 9	sc-56076	1:500	Santa Cruz Biotechnology
Anti-Cyclin A	PA5-36048	1:500	Invitrogen, Thermo Fisher Scientific
Anti-Cyclin D1	MA514512	1:1000	Invitrogen, Thermo Fisher Scientific
Anti-Cycline E	sc-247	1:500	Santa Cruz Biotechnology
Anti-Cytocrome C	sc-13156	1:500	Santa Cruz Biotechnology
Anti-ERK 1/2	sc-154	1:1000	Santa Cruz Biotechnology
Anti-GAPDH	60004-1-Ig	1:5000	ProteinTech
Anti-p21	MA531479	1:1000	Invitrogen, Thermo Fisher Scientific
Anti-p27	sc-528	1:500	Santa Cruz Biotechnology
Anti-p38	AH01202	1:1000	Invitrogen, Thermo Fisher Scientific
Anti-p53	sc-126	1:500	Santa Cruz Biotechnology
Anti-PARP-1	sc-8807	1:500	Santa Cruz Biotechnology
Anti-p-ERK 1/2	MA515173	1:1000	Invitrogen, Thermo Fisher Scientific
Anti-p-p38	9211S	1:1000	Cell Signaling Technology
Anti-Survivin	sc-10811	1:500	Santa Cruz Biotechnology

1.7 Proliferation assay

Cells isolated from secondary generation mammospheres and adherent culture were seeded in flat-bottom 96-well plates in 100 µl of full media (10×10^3 cells/well). After 24h cells were treated with adiponectin for 24, 48, 72h in phenol-red free medium containing 10% dextran charcoal-stripped FBS. At desired endpoint, wells were emptied and plates were stored at -80°C until ready to scan. For proliferation assay, plates were thawed to room temperature and were added 100 µl distilled water per well. Subsequently, plates were incubated at 37°C for 1h and then placed at -80°C until frozen. After the thaw to RT, it has been added 100 µl of Hoechst dye reagent (ThermoFisher Scientific), diluted in TNE buffer according

manufacturer's instructions, and fluorescence was measured using excitation and emission filters at 360 nm and 460 nm, respectively.

1.8 Annexin V/PI assay

Early apoptotic events were evaluated using Dead Cell Apoptosis Kit with Annexin V FITC & Propidium Iodide for Flow Cytometry (Invitrogen). Cells obtained from secondary generation mammospheres were pelleted, washed in cold PBS and resuspended in 1X annexin-binding buffer provided by the kit. Once established cell density, 100 µl of cell suspension (containing 1×10^6 cells/ml of buffer) were incubated with 2 µl of Annexin V and 5 µl of PI for 15' in the dark at RT. After the incubation period, 400 µl of Binding Buffer 1X were added to each tube and the samples were analyzed by CytoFLEX flow cytometry (Beckman-Coulter). The data were acquired using CytExpert Beckman Coulter software.

1.9 Reactive oxygen species (ROS) assessment

ROS production was measured using the chloromethyl derivative of 2',7′-dichlorodihydrofluorescein diacetate CM-H2DCFDA (ThermoFisher Scientific), a cell-permeable probe that is non-fluorescent until oxidation within the cell. Secondary mammosphere-derived cells were collected and incubated with CM-H2DCFDA (diluted in PBS to a final concentration of 10 µM) at 37°C, for 30-40'. Subsequently, the stained cells were harvested by centrifugation and maintained in a fresh medium, and incubated at 37°C for 20'. The ROS levels were estimated by using the mean fluorescent intensity of the viable cell population. The results were

obtained with CytoFLEX flow cytometry (Beckman-Coulter) and analyzed using CytExpert Beckman Coulter software.

1.10 Mitochondrial membrane potential assay

Mitochondrial membrane potential was measured by FACS analysis (CytoFLEX Beckman-Coulter) in cells stained with MitoTracker® Orange CMH2TMRos (ThermoFisher Scientific). Cells obtained from secondary generation mammospheres were collected and incubated with CMH2TMRos (diluted in PBS to a final concentration of 1 μM) for 30' at 37°C. Cells were then maintained in a fresh medium at 37°C for 20' before the flow cytometry analysis. The results obtained were analyzed using CytExpert Beckman Coulter software.

1.11 Proteomic analysis

Filter Aided Sample Preparation (FASP) digestion. 50 μg of whole cell extract (100 μl), obtained from secondary generation mammospheres, were dissolved in SDS 1%, 50 mM DTT, and 50 mM Tris HCl pH 8.0 and heated at 95°C for 10'. Next, samples were transferred to Microcon 10 kDa cutoff filters (Merck), centrifuged at 14,000 × g for 15' and then washed two times with 200 μl of urea buffer (8M urea, 100 mM Tris buffer, pH 8.0). Subsequently, 100 μl of 50 mM iodoacetamide (IAA) in 8 M urea buffer was added to carry out Cysteine alkylation and samples were incubated for 20' in dark at RT. After centrifugation for 15', filters were additionally washed two times with urea buffer and two times with 50 mM triethylammonium bicarbonate (TEAB). Then, 500 ng of trypsin were added to each sample for digestion at 37°C ON. Following digestion 140 μl of H_2O were

added and the samples were centrifuged at 14,000 × g for 20' to recover 180-200 µl of peptides.

Peptides purification. Strong cation exchange (SCX) StageTips method was performed to purify 5 µg of peptides. 1 mm^3 of EmporeTM-3M SCX resin (Merck) was used as stationary phase while the digested samples were acidified by adding 80% acetonitrile-0.5% formic acid (Solution W2). The StageTip was conditioned by adding 20% acetonitrile-0.5% formic acid (Solution W1) and 50 µl of W2. Peptides were loaded by slowly letting the fluid pass through one plug using a benchtop centrifuge. After two washes with 50 µl of Solution W2 and two washes with 50 µl of Solution W1, 10 µl of 500 mM ammonium acetate, 20% acetonitrile was added to eluate peptides.

LC-MS/MS analysis. A Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) system consisting of an Easy nLC-1000 chromatographic instrument connected to a Q-Exactive-Orbitrap mass spectrometer (both from ThermoFischer Scientific) was used to carry out LFQ (Label Free Quantification) analysis. For all the LC-MS/MS analyses the flowrate was 0.3 µl/min. A binary gradient elution was used for transporting the solution into the mass spectrometer with two mobile phases consisting of 98% water, 0.1% formic acid in 2% acetonitrile (mobile phase A) and 20% water, 80% acetonitrile, and 0.1% formic acid (mobile phase B) (ThermoFischer Scientific). For mass spectrometer settings, data-dependent analysis (DDA) was used as a method for spectrum identification. All chemicals used in the experiments described were purchased from Merck unless otherwise specified.

Data analysis. Andromeda search engine integrated into MaxQuant software (2.0.1.0 version) was used to analyze Raw MS data. The MS/MS spectra were searched against a human proteome database. For Label-Free (LF) samples the following settings were used: fixed modifications: carbamidomethyl (C); variable modification: oxidized methionine (M) and acetyl (N-terminus). Unique peptides were used for protein identification.

Differentially expressed proteins (DEPs), presenting an FDR ≤ 0.05 and an absolute log2FC higher than 2, were selected for further heatmap images and functional analyses. Perseus software (2.0.11 version) was used to perform the statistical analysis of MaxQuant output. LFQ intensity of proteins were imported and contaminants, such as proteins only identified by site modifications which may not be accurate, were filtered. Moreover, data were transformed into logarithmic scale (log2). Proteins quantified in less than two replicates belonging to at least one group were discarded. A two-sample t-test was carried out to compare the untreated and adiponectin-treated mammospheres, identifying proteins that are significantly differentially expressed. Hierarchical clustering was performed by generating z-scores of normalized LFQ intensities. Values were clustered using the Euclidean distance method and a heatmap was generated to visualise the intensity levels of protein expression among mammospheres obtained from WT and TR MCF-7 cells untreated or treated with adiponectin. Venn diagrams were created by using https://www.interactivenn.net/ web-tool to represent the significantly DEPs in the WT and TR MCF-7 mammospheres untreated or treated with adiponectin. Finally, pathway analysis was performed using MetaCore software (Clarivate Analytics), a knowledge database suitable for pathway analysis of experimental data and gene

lists. The DEP lists with adjusted P-value and log2- transformed were uploaded with a threshold of q-value < 0.1. The results of the enrichment analysis provided pathway maps, which were determined to be of statistical significance using an FDR < 0.05.

1.12 Wound healing assay

Cells obtained from monolayer cultures and the disaggregation of secondary generation mammospheres were seeded at a density of 1.5×10^5 cells/well in 6-well tissue culture plates until 100% confluency was reached. A scratch (wound) was made using a 10 µl pipette tip, and phenol red-free and serum-free media in the absence or presence of adiponectin was added. Each scratch was immediately imaged (at 0h) 3 times at different points using phase-contrast microscopy at 4x magnification. Images were captured using a sCMEX-3 microscope camera (Euromex) and ImageFocus Alpha software. Then, cells were maintained at 37°C, 5% CO_2, and images were captured at 6, 24 and 48h. The wounding area was measured and analyzed using Image J Software.

1.13 Statistical analysis

Each data point represents the mean ± S.D. of at least 3 independent experiments. Data were analyzed by Student's T test using the Prism 7.0 (GraphPad Software) software program. $p<0.05$ was considered as statistically significant.

Results

1. Adiponectin induces an enrichment of stem subpopulation in TR MCF-7 mammospheres

To evaluate the ability of low adiponectin level to influence BCSCs activity in hormone resistance, we used as experimental model hormone-responsive and tamoxifen-resistant breast cancer cells (WT and TR MCF-7 cells, respectively), treated with adiponectin 5 μg/ml (A5), and grown as mammospheres.

This culture condition allowed to enrich, propagate and characterize breast cancer cells with stem-like phenotype, able to escape anoikis in an anchorage-independent system[192].

The number of spheres formed during serial passages in suspension has been used to estimate mammospheres formation capability and self-renewal ability, hallmarks of CSCs. Our results showed that, in primary generation, adiponectin-treated TR MCF-7 cells, grown in anchorage-independent conditions, displayed a higher efficiency of mammospheres formation than WT MCF-7 cells (Figure 11A). Cells obtained from the dissociation of primary generation mammospheres were re-plated in the same culture conditions to carry out secondary generation mammospheres. Our experiments demonstrated that in tamoxifen-resistance counterpart adiponectin increased mammosphere forming efficiency (MFE) even more in secondary generation compared to primary generation (Figure 11B). On the contrary, in hormone-responsive cells adiponectin was not able to induce a significant increase of MFE (Figure 11B).

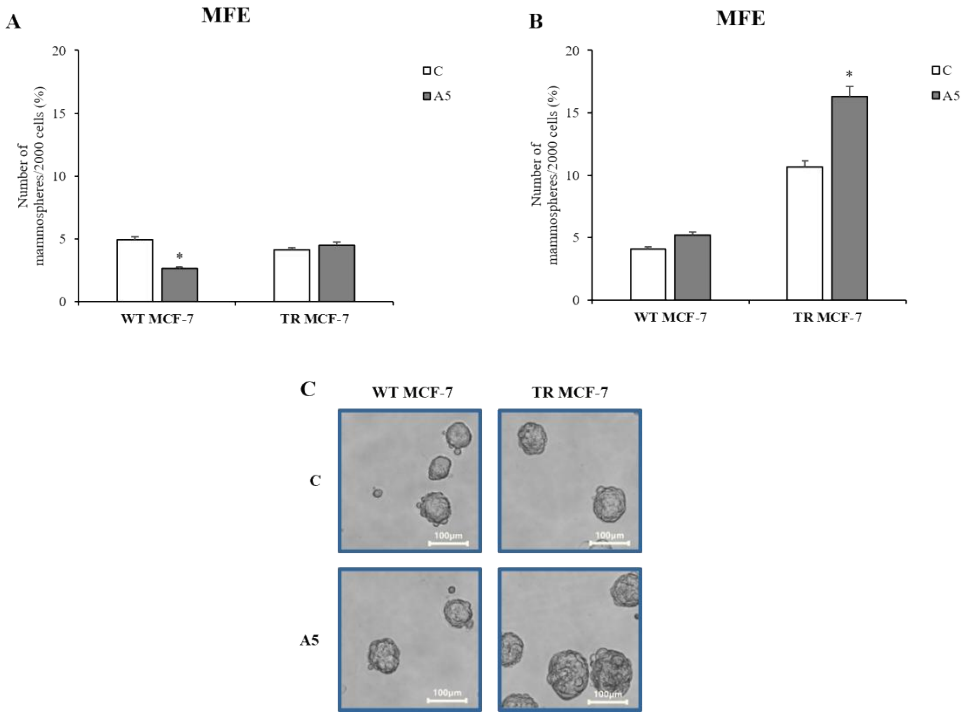
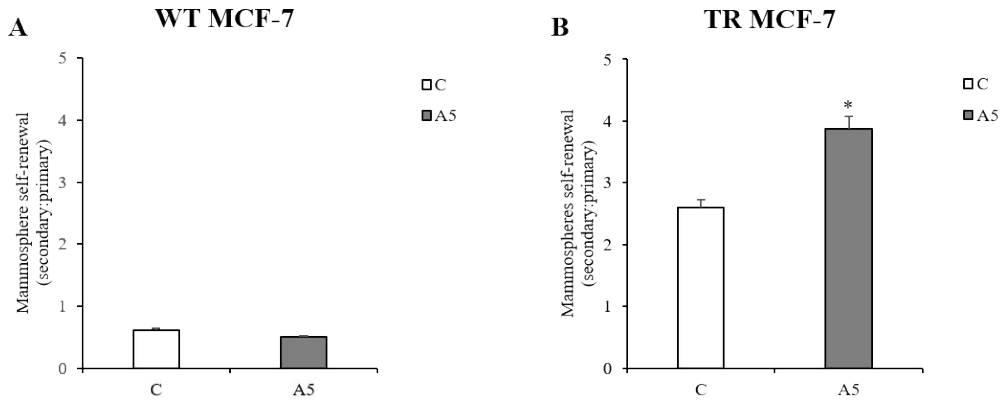


Figure 11. Mammosphere Forming Efficiency (MFE) of WT and TR MCF-7 cells. MFE evaluated in WT and TR MCF-7 (**A**) primary and (**B**) secondary generation in the absence (control, C) or presence of adiponectin 5 µg/ml (A5). The histograms represent the mean of ± S.D. of three separate experiments. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C. (**C**) Representative bright field images of secondary WT and TR MCF-7 mammospheres acquired using a sCMEX-3 microscope camera (Euromex, Spain) and ImageFocus Alpha software. Scale bar=100 µm.

Consistent with these data, self-renewal assessed as the ratio between the number of secondary and primary generation spheres, revealed a greater capability for self-renewal in TR MCF-7 cells compared to WT MCF-7 cells, which was more evident upon adiponectin treatment (Figure 12A and B).



MCF-7 WT		
	MAMMOSPHERES NUMBER	
	C	A5
Primary generation	101,833	52,167
Secondary generation	62,66667	26,0
SELF-RENEWAL	**0,615384648**	**0,498402556**

TR MCF-7		
	MAMMOSPHERES NUMBER	
	C	A5
Primary generation	81,833	84,0
Secondary generation	213,0	325,8
SELF-RENEWAL	**2,603**	**3,879**

Figure 12. Mammosphere Self-Renewal (MSR) estimated in adiponectin-treated WT and TR MCF-7 cells. MSR estimated in (**A**) WT and (**B**) TR MCF-7 in the absence (control, C) or presence of adiponectin 5 µg/ml (A5). The histograms represent the mean of ± S.D. of three separate experiments (upper panel). Representative value of MSR (bottom panel). Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C.

Next, to characterize cell subpopulations that compose secondary generation mammospheres, the percentage of cells expressing membrane proteins CD44 and CD24, markers that have been recognized to correspond to more mesenchymal- and epithelial-like CSCs respectively[193], was evaluated. Flow cytometry analysis highlighted that mammospheres obtained from adiponectin-treated TR MCF-7 cells were enriched in $CD44^+/CD24^-$ subpopulation. In contrast, $CD44^+/CD24^-$ ratio was reduced in WT MCF-7 mammospheres upon adiponectin exposure (Figure 13A). These findings were further validated by qRT-PCR analysis that showed the up-regulation of CD44 mRNA expression levels, concomitant to the down-regulation of CD24 mRNA levels, in mammosphere-derived TR MCF-7 cells, less evident in WT counterpart (Figure 13B).

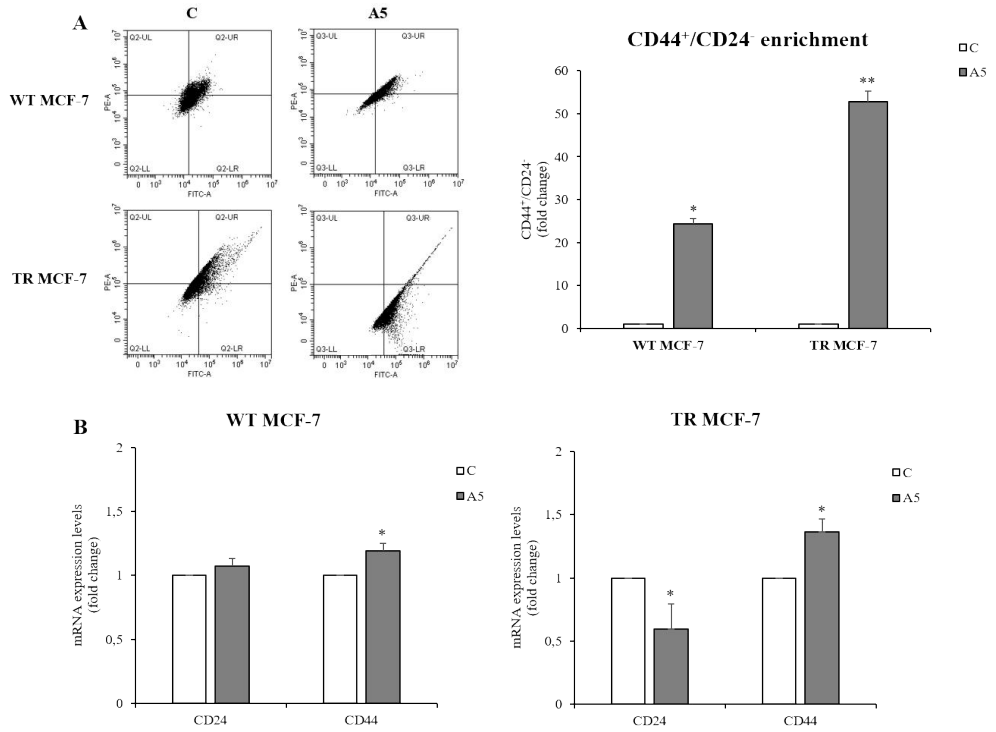


Figure 13. CD44$^+$/CD24$^-$ BCSC subpopulation enrichment in WT and TR MCF-7 mammospheres. (**A**) Flow cytometry analysis of CD44 and CD24 expression in secondary WT and TR MCF-7 mammospheres untreated (Control, C) or treated with adiponectin 5 μg/ml (A5). Representative flow cytometry data is shown (left panel). The values are representative of CD44 and CD24 positive cells percentage expressed as fold change (right panel). (**B**) qRT-PCR in WT and TR MCF-7 secondary generation mammosphere-derived cells to analyze CD44 and CD24 mRNA levels. 18S mRNA was used to normalize the variability in template loading. The histograms represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

To better characterize the enrichment of CSCs in WT and TR MCF-7 cells derived from adiponectin-treated secondary generation mammospheres, we evaluated the expression levels of ALDH1, a functional marker for BCSCs, associated with metastasis, poor prognosis and clinical outcomes in breast cancer patients[194]. FACS analysis evidenced that in WT MCF-7 cells, grown as mammospheres, adiponectin reduced ALDH1 expression, while a significant increase was observed in TR MCF-7 cells (Figure 14).

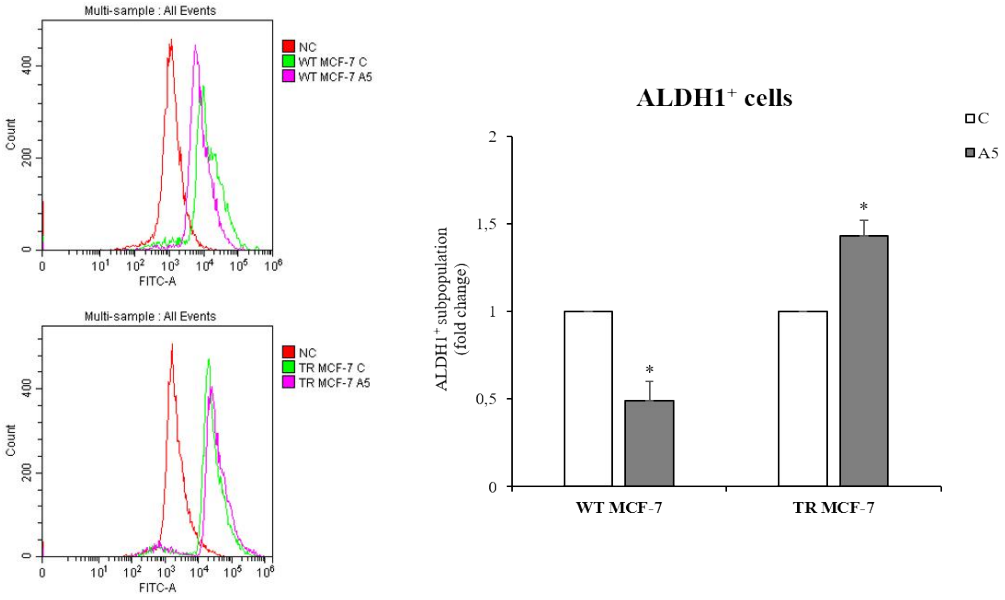


Figure 14. Flow cytometry analysis of ALDH1 expression in WT and TR MCF-7 mammospheres. Representative plots of flow cytometry (left panel) and related quantification (right panel) of ALDH1$^+$ cells derived from secondary generation WT and TR MCF-7 mammospheres untreated (control, C) or adiponectin-treated (A5). The histograms represent the means ± S.D. of three different experiments. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C.

Therefore, the results obtained demonstrated that adiponectin promoted classical stem-like features in hormone-resistant breast cancer cells.

2. Effect of adiponectin on the expression of stemness and mesenchymal markers in hormone-resistant mammospheres

The phenotypic characterization led us to investigate whether adiponectin may affect the expression levels of the main biomarkers involved in the maintenance of BCSCs phenotype. To this aim, we performed qRT-PCR analysis on total mRNA extracted from the secondary generation spheres. The results showed a significant up-regulation of mRNA expression levels of genes that play a key role in stem cell biology, such as Oct-4, Notch (isoforms 1, 2, 3, 4), Sox (isoforms 2, 4, 9) and

SMAD4 in adiponectin-treated TR MCF-7 cells. On the contrary, an opposite trend was observed in WT MCF-7 cells (Figure 15).

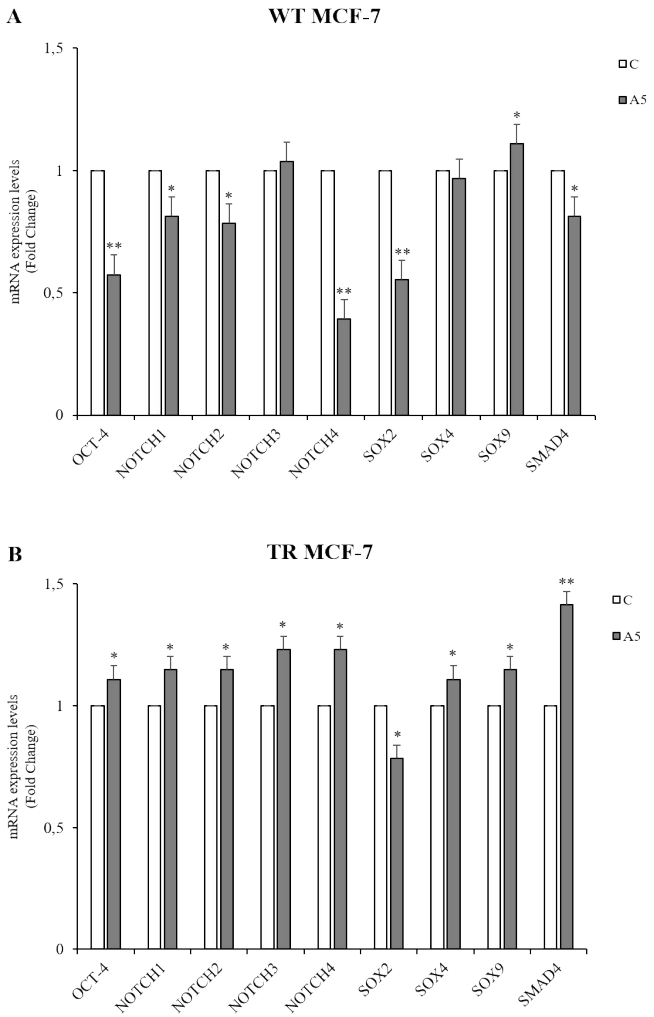


Figure 15. Identification of cancer stem-like phenotype according to stem cell markers in WT and TR MCF-7 mammospheres. qRT-PCR validation of a subset of genes involved in stemness in secondary (**A**) WT and (**B**) TR MCF-7 mammospheres untreated (Control, C) or treated with adiponectin 5 μg/ml (A5). 18S mRNA was used to normalize the variability in template loading. The histograms represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs,* **p<0.01 vs C.

Since EMT supports tumor cells to acquire mesenchymal properties and confers the capability to cancer cells to enter CSC status, we evaluated if adiponectin may promote the transition towards a mesenchymal state in endocrine-resistance BCSCs. qRT-PCR analysis demonstrated that in TR MCF-7 cells, grown as

mammospheres, adiponectin induced a reduction of E-cadherin levels, a protein involved in homotypic adhesion and main epithelial marker, concomitant with an increased expression of mesenchymal markers, such as Vimentin, α-SMA and TWIST (Figure 16). In contrast, in WT MCF-7 cells adiponectin enhanced E-cadherin expression and induced a slight increase of mesenchymal marker levels, although in a lesser extent compared to TR MCF-7 cells (Figure 16).

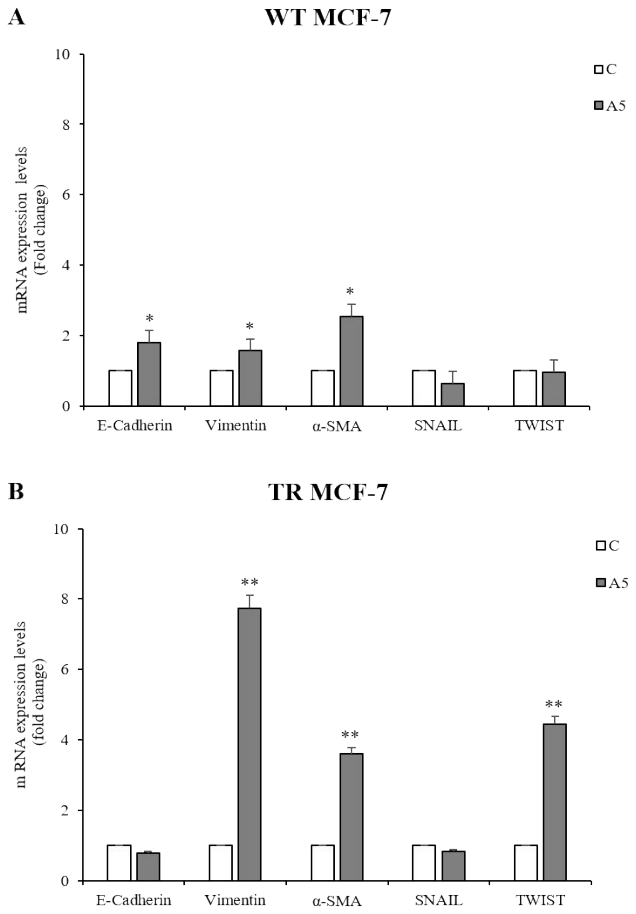


Figure 16. EMT marker profiling of WT and TR MCF-7 mammospheres. Relative expression of the mRNAs encoding epithelial (E-cadherin) and mesenchymal (Vimentin, α-SMA, SNAIL and TWIST) markers evaluated by qRT-PCR in secondary (**A**) WT and (**B**) TR MCF-7 mammospheres untreated (Control, C) or treated with adiponectin 5 μg/ml (A5). 18S mRNA was used to normalize the variability in template loading. The histograms represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

Overall, these findings supported the role of adiponectin as a possible mediator of CSCs activity in hormone-resistant breast cancer cells.

3. Cell cycle distribution and proliferation are regulated by adiponectin in TR MCF-7 mammospheres

Cell cycle progression is commonly deregulated in breast cancer cells with stem-like phenotype and this feature allows them to play a pivotal role in tumor initiation, progression and recurrence. Thus, FACS analysis was performed in order to explore the effect of adiponectin on BCSCs distribution in cell cycle phases. The results revealed a cell cycle arrest in G0/G1 phase induced by adiponectin in TR MCF-7 cells, which was not evident in WT MCF-7 cells (Figure 17).

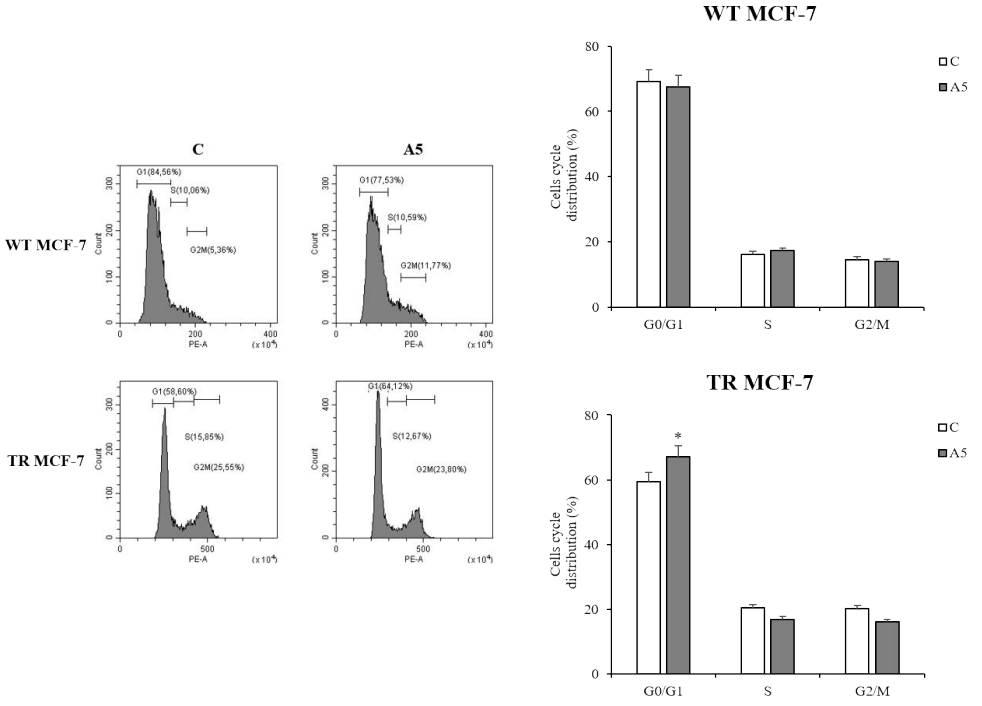


Figure 17. Cell cycle analysis of WT and TR MCF-7 mammosphere-derived cells. Representative flow cytometry plots (left panel) of cell cycle distribution in WT and TR MCF-7 secondary generation mammosphere-derived cells untreated (Control, C) or treated with A5. Cell quantitation in cell cycle phases (right panel). The histograms represent the means ± S.D. of three different experiments. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C.

To further elucidate the mechanisms by which adiponectin regulates cell cycle phases in mammosphere-derived WT and TR MCF-7 cells, we assessed adipokine influence on the expression of cell cycle-related proteins. The results by WB analysis showed unchanged levels of Cyclin D1, protein involved in G0/G1 phase transition, Cyclin E, regulating G1/S phase transition, and Cyclin A, driving the entry in G2 phase, in both cell lines (Figure 18). Noteworthy, in adiponectin-treated TR MCF-7 mammospheres it has been detected an increased expression of p21 and p27, cyclin-dependent kinase inhibitors involved in cell cycle, not observed in WT MCF-7 mammospheres (Figure 18).

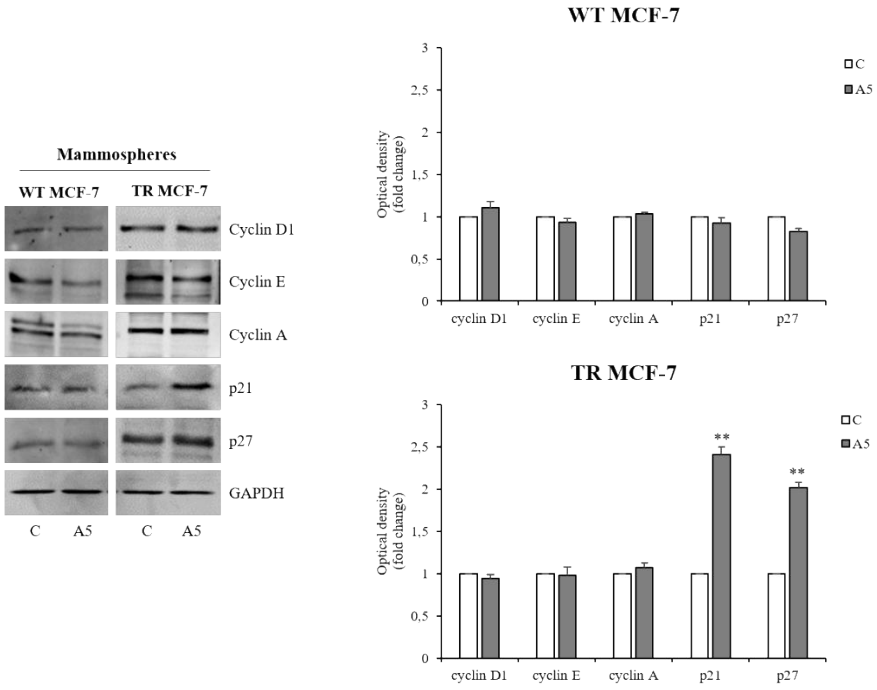


Figure 18. Adiponectin affects cell cycle progression in TR MCF-7 mammospheres. Western blotting of Cyclin D1, Cyclin E, Cyclin A, p21 and p27 expression in WT and TR MCF-7 secondary generation mammospheres untreated (Control, C) or treated with adiponectin 5 μg/ml (A5). GAPDH was used as loading control. Images are representative of three different experiments (left panel). The histograms represent the means ± S.D of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold change of the C assumed as 1 (right panel). Data were analyzed by Student's T test using the GraphPad Prism 7 software program. **p<0.01 *vs* C.

Moreover, qRT-PCR analysis evidenced a down-regulation of Cyclin D1 mRNA levels, concomitant with an up-regulation of p21 mRNA content in adiponectin-treated TR MCF-7 mammospheres (Figure 19).

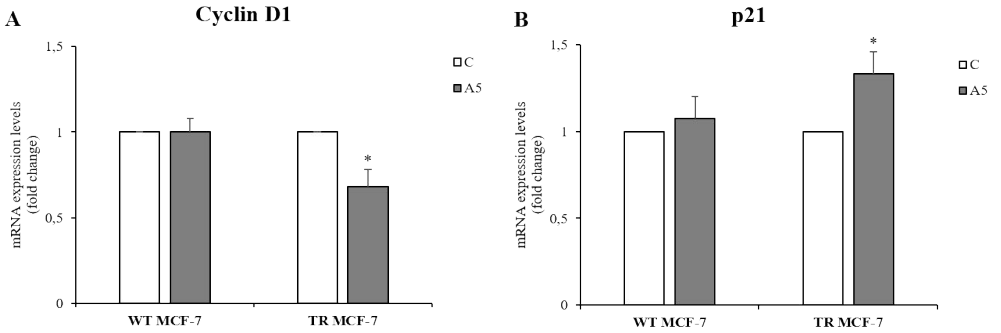


Figure 19. Effects of adiponectin on cell cycle regulators of G0/G1 phase transition. Relative expression of the mRNAs encoding Cyclin D1 (**A**) and p21 (**B**) markers estimated by qRT-PCR in WT and TR MCF-7 mammospheres of secondary generation, untreated (control, C) or treated with adiponectin 5 μg/ml (A5). 18S mRNA was used to normalize the variability in template loading. The histograms represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *$p<0.05$ *vs* C.

These data demonstrated that adiponectin resisted cell cycle progression, preventing the entry in S phase, in TR MCF-7 mammospheres.

Cell cycle arrest in G0/G1 phase induced by adiponectin in hormone-resistant BCSCs led us to investigate the ability of adiponectin to modulate the growth kinetics of WT and TR MCF-7 cells isolated from secondary generation mammospheres. After 48h of treatment, adiponectin induced a significant decrease in cell viability of mammosphere-derived TR MCF-7 cells, while the proliferation rate of WT MCF-7 mammospheres was increased (Figure 20). To corroborate these findings, we evaluated the mRNA expression levels of Ki67, marker used as index of proliferative capacity, in mammosphere-derived cells. Interestingly, qRT-PCR

showed an increase in Ki67 levels in adiponectin-treated WT MCF-7 cells compared to untreated counterpart. In contrast, it has been evidenced that adiponectin decreased the expression levels of Ki67 in TR MCF-7 cells (Figure 20). These results confirmed the enrichment of stem cells in TR MCF-7 cells cultured as mammospheres.

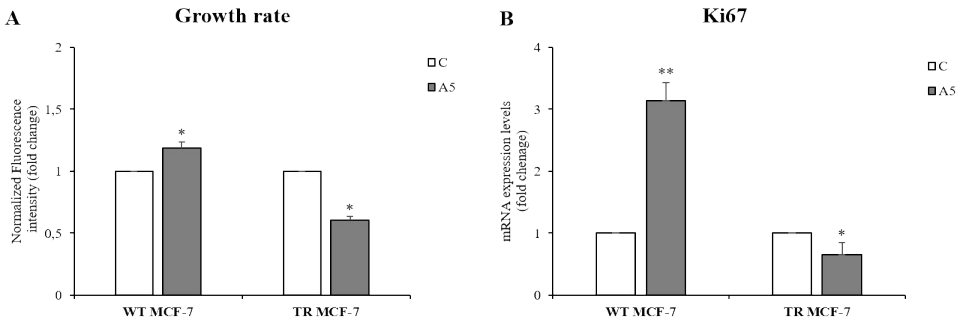


Figure 20. Effect of adiponectin on breast cancer cell growth. (**A**) FluoReporter Blue Fluorometric dsDNA Quantitation Kit was used to assess the growth rate in secondary generation mammosphere-derived WT and TR MCF-7 cells untreated (control, C) or treated with A5. Cell proliferation is expressed as fold change. (**B**) qRT-PCR for Ki67 mRNA expression in WT and TR MCF-7 mammosphere-derived cells. 18S mRNA was used to normalize the variability in template loading. The histograms represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

4. Adiponectin-treated TR MCF-7 mammospheres escape apoptosis

The BCSCs arrest in G0/G1 phase of the cell cycle suggested their ability to remain in a quiescent state and evade cell death process. To ascertain if in the latter condition BCSCs switch towards quiescent state or apoptosis, we first investigated the effects of adiponectin on the early apoptotic events.

To this aim, Annexin V assay was used to elucidate adiponectin impact on apoptotic process in WT and TR MCF-7 cells isolated from secondary generation

mammospheres. As shown in Figure 21A, the mammosphere-forming TR MCF-7 cells displayed a significant apoptotic event reduction upon adiponectin treatment. In contrast, no change in apoptosis was detected in the adiponectin-treated WT counterpart.

To corroborate the reduction of apoptosis induced by adiponectin in hormone-resistant BCSCs, we investigated the expression of the main Bcl-2 family-related proteins, which play pivotal roles in regulating apoptosis.

Our results showed increased levels of the anti-apoptotic proteins Bcl-2 and Survivin upon adiponectin treatment in TR MCF-7 mammospheres. This was concomitant with reduced levels of Bid, tBid and Bax, pro-apoptotic molecules (Figure 21B). In contrast, in WT MCF-7 mammospheres adiponectin produced a slight increase in the expression of the apoptosis-regulating proteins Bid and Bax (Figure 21B).

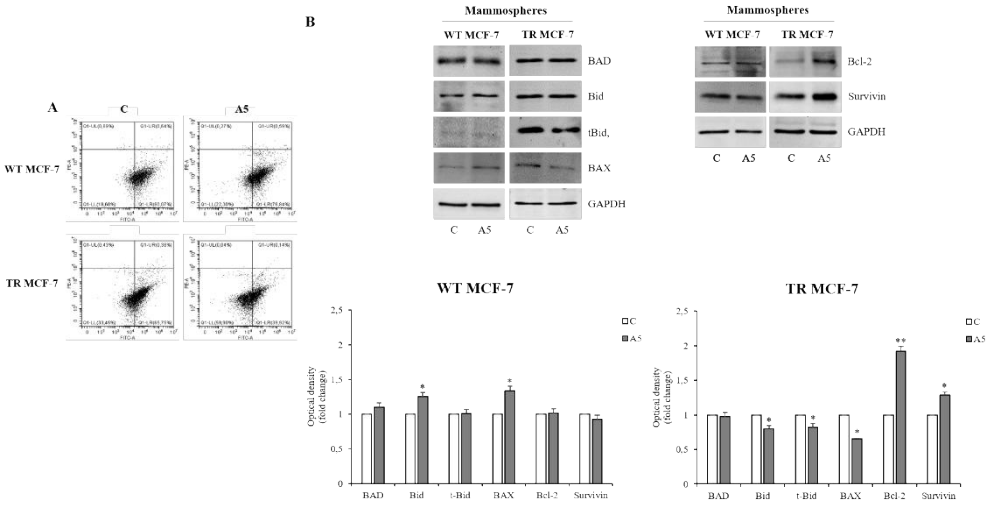


Figure 21. Adiponectin affects apoptosis in TR MCF-7 mammospheres. (**A**) Secondary generation WT and TR MCF-7 mammospheres untreated (Control, C) or adiponectin-treated (A5) were stained with Annexin V-FITCH and Propidium iodide (PI). Apoptosis was assessed by flow cytometry analysis. The four quadrants represent living cells (lower left, Annexin V-/PI-), early apoptotic (lower right, Annexin V+/PI-), late apoptosis (upper right Annexin V+/PI+), necrotic (upper left Annexin V-/PI+) stages. The graphic is representative of three different experiments. (**B**) BAD, Bid, tBid, Bax, Bcl-2 and Survivin evaluated by western blotting in protein extracts from secondary generation WT and TR MCF-7 mammospheres. GAPDH was used as loading control. The histograms represent the means ± S.D of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold change of the C assumed as 1. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

A further support of the reduction of apoptotic event displayed by TR MCF-7 mammospheres, came from the property of Bcl-2 family members to control the release of cytochrome C and the activation of Caspase-9 and -3, which in turn induce inhibitory PARP-1 proteolytic cleavage, driving apoptosis[195]. Based on these findings, western blotting analysis performed in adiponectin-treated TR MCF-7 mammospheres demonstrated reduced cytochrome C levels with consequent loss of PARP-1 caspase-mediated inactivation and concomitant p53 decreased expression (Figure 22).

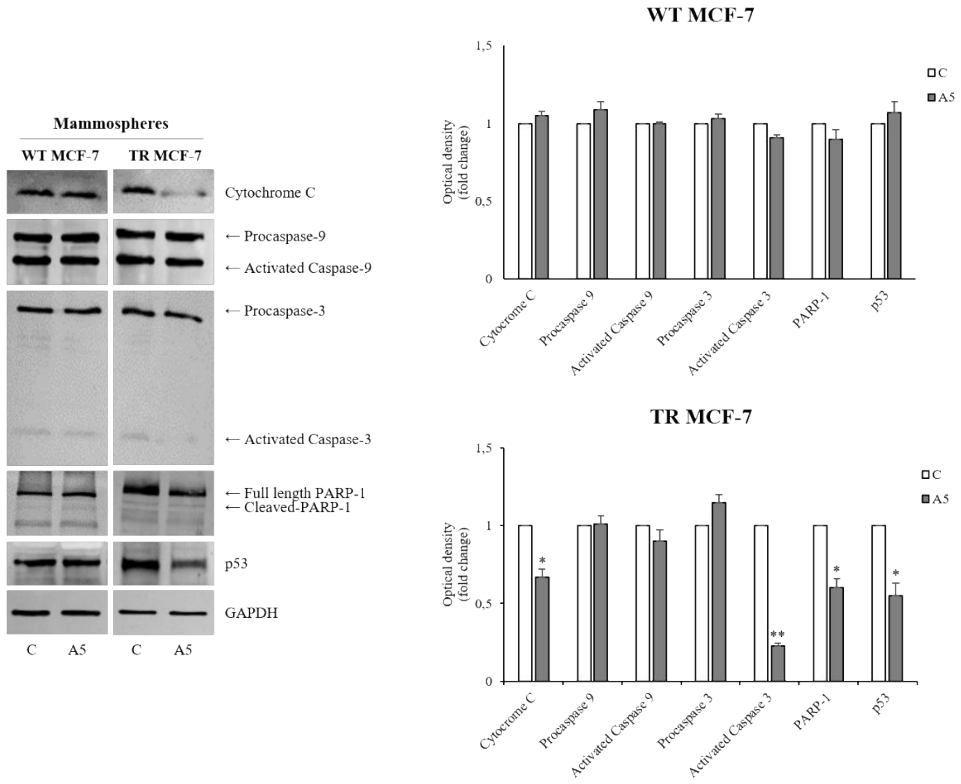


Figure 22. Adiponectin impairs cytochrome C release. Representative images of western blotting analysis of Cytochrome C, Caspase-9, Caspase-3, PARP-1 and p53 expression in WT and TR MCF-7 mammospheres of secondary generation untreated (Control, C) or treated with adiponectin 5µg/ml (A5). GAPDH was used as loading control. The histograms represent the means ± S.D of three different experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold change of the C assumed as 1. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

It has been previously documented that an excessive ROS production may modulate intracellular apoptotic event. This has given the rationale to investigate ROS levels in TR MCF-7 cells.

Flow cytometry analysis showed that adiponectin induced a reduction of intracellular ROS levels, more evident in mammosphere-derived TR MCF-7 cells compared to WT cells (Figure 23).

This reasonably suggested that ROS level drop, induced by adiponectin treatment, may be crucial in maintaining stem-like phenotype in hormone-resistant BCSCs.

The reduced ROS production maintains mitochondrial membrane potential, preventing the release of cytochrome C, in such way reducing the intracellular apoptotic event.

Our data evidenced that mitochondrial membrane potential was slightly enhanced in both cell lines upon adiponectin, suggesting a healthy mitochondrial functioning and confirming apoptosis escape (Figure 23).

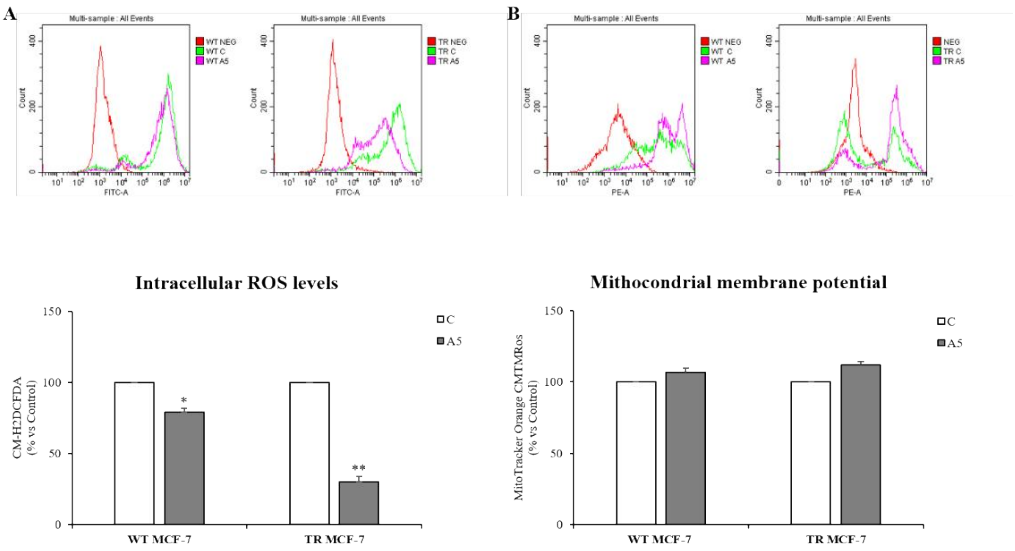


Figure 23. ROS production is reduced in adiponectin-treated mammospheres. Representative flow cytometry overlay images of ROS intracellular levels (**A**) and Mitochondrial membrane potential (**B**) in secondary WT and TR MCF-7 mammospheres untreated (Control, C) or treated with adiponectin 5µg/ml (A5) (upper panel). Untreated cells were used as negative control. Data are shown as probe fluorescence normalized on viable cell number and expressed as percentage *vs* C (**A** and **B**) (bottom panel). The histograms represent the means ± S.D. of three different experiments. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C, **p<0.01 *vs* C.

5. Proteomic and enrichment analysis in MCF-7 mammospheres

By Mass Spectrometry (MS-Based Proteomics) we investigated the modulatory role of adiponectin in proteome profile in WT and TR MCF-7 mammospheres.

First, Hierarchical clustering was carried out based on the protein intensity. The obtained heat map showed difference in protein expression between hormone-responsive and hormone-resistant breast cancer mammospheres upon adiponectin exposure (Figure 24A). In addition, we used the Venn diagram web-tool (https://interactivenn.net) to evaluate the adiponectin effect on protein expression in WT and TR MCF-7 mammospheres. A total of 132 and 128 Differentially Expressed Proteins (DEPs) were identified in WT and TR MCF-7 mammospheres, respectively. According to their log2FC and p-values, it has been identified 77 up-regulated and 55 down-regulated proteins in WT MCF-7 mammospheres. On the other hand, in TR MCF-7 mammospheres 88 up-regulated and 40 down-regulated proteins were noticed. Moreover, Venn diagrams evidenced 15 overlapped DEPs, including 8 up-regulated and 2 down-regulated proteins (Figure 24B).

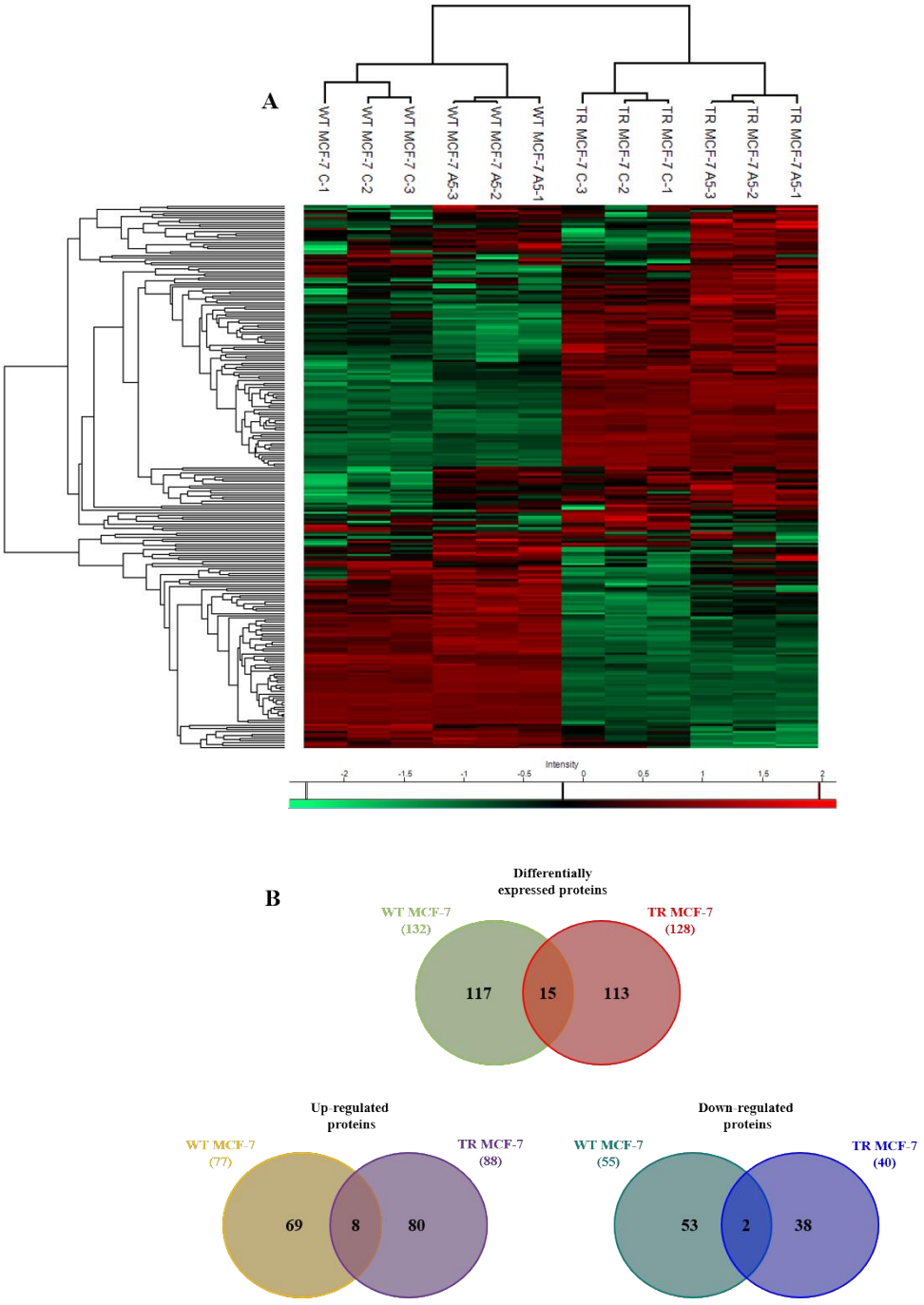


Figure 24. Adiponectin effect on global changes of protein expression in WT and TR MCF-7 mammospheres. (**A**) Statistical analysis on the MS data was performed by Perseus software. Hierarchical clustering and heatmap of protein intensity in WT and TR MCF-7 mammospheres untreated (Control, C) or treated with adiponectin 5µg/ml (A5) were shown. Normalization was based on z-scores and the data for each row was normalized by the mean value calculated for the same row. The intensity value depicts the direction of protein expression as up-regulated (red) or down-regulated (green). Three repeats per condition were performed. (**B**) Venn diagrams showed the overlap of identified proteins in the three biological replicates, obtained from WT and TR MCF-7 mammospheres.

To understand the role of DEPs and the pathway maps in the WT and TR MCF-7 mammospheres, we used MetaCore^{TM} software (Clarivate Analytics) to perform enrichment analysis using a widely known database for protein-protein signaling. Enrichment analysis of the DEP datasets identified the top 10 enriched pathways, and the maps in both cell lines. Among them, we discovered that, in line with the above reported results, apoptosis-related pathway played a critical role in hormone-resistant breast cancer cells, such as "Apoptosis and survival Granzyme B signaling", "Apoptosis and survival caspase cascade", "Apoptosis and survival TNFR1 signaling" pathway maps (Figure 25A). Particularly, Figure 25B displayed the pictorial representation of the fourth top-scored pathway map resulting from the distribution of protein enrichment in TR MCF-7 mammospheres. The well-characterized proteins were presented as individual symbols and the data from all the experiments were shown and linked on the maps as thermometer-like symbols. Interestingly, among the down-regulated proteins, labelled by blue thermometer, Bid and tBid well fit with the expression levels previously validated by western blotting analysis (Figure 21B). Overall, molecular and omic analysis demonstrated the critical role of adiponectin in reducing apoptosis in hormone-resistant BCSCs.

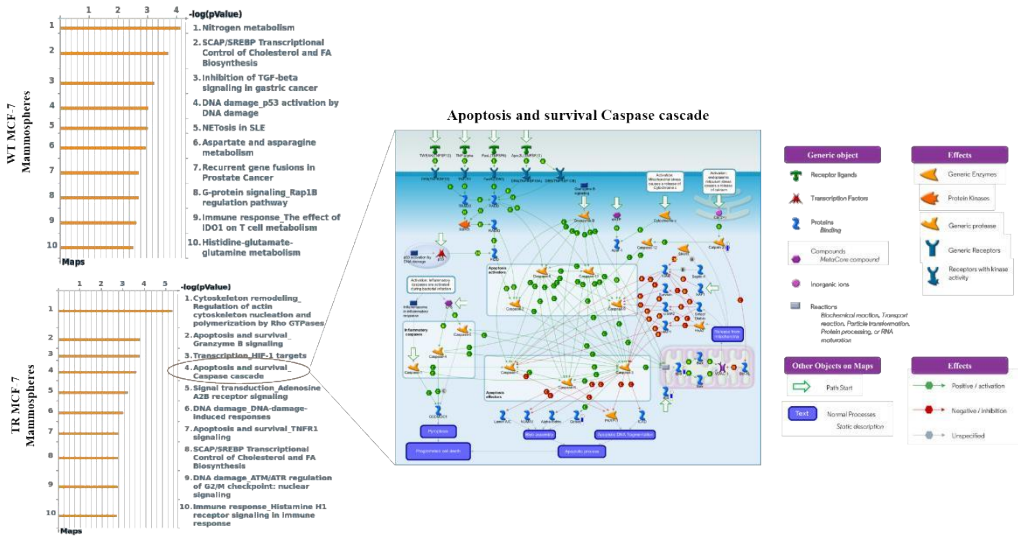


Figure 25. Pathway maps of MetaCore™ analysis. Left panel shows the top 10 pathway profiles in secondary WT and TR MCF-7 mammospheres untreated (control, C) or treated with adiponectin 5µg/ml (A5). Sorting is done for the "Statistically significant Maps". Canonical pathway maps representing a set of signaling maps covering human in a comprehensive way. All maps are created by Clarivate scientists by a high-quality manual curation process based on published peer-reviewed literature. Right panel displays the "Apoptosis and survival Caspase cascade" map, the fourth scored map obtained on the basis of the enrichment distribution sorted by "Statistically significant Maps" set. Proteins on the map are indicated by different symbols representing the functional class of the protein reported in the legend. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the proteins.

6. Does adiponectin induce quiescence in BCSCs?

The evidence that, in hormone-resistant mammospheres, adiponectin induced G0/G1 cell cycle arrest, characterized by $Ki67^{low}$ and $p27^{high}$, apoptosis escape and decreased ROS level, led us to speculate its involvement in maintaining a nonproliferating phenotype, denoting a quite close quiescent state.

Thus, we investigated the modulation of different signaling pathways related to the quiescent phenotype. We detected a significant decrease in p-ERK/ERK ratio, concomitant with an increase in p-p38/p38 ratio (Figure 26), coherent with a slower proliferation rate in TR MCF-7 mammospheres (Figure 20A). As expected, an

opposite trend was observed in WT MCF-7 cells (Figure 26), according to the high growth rate above reported (Figure 20A).

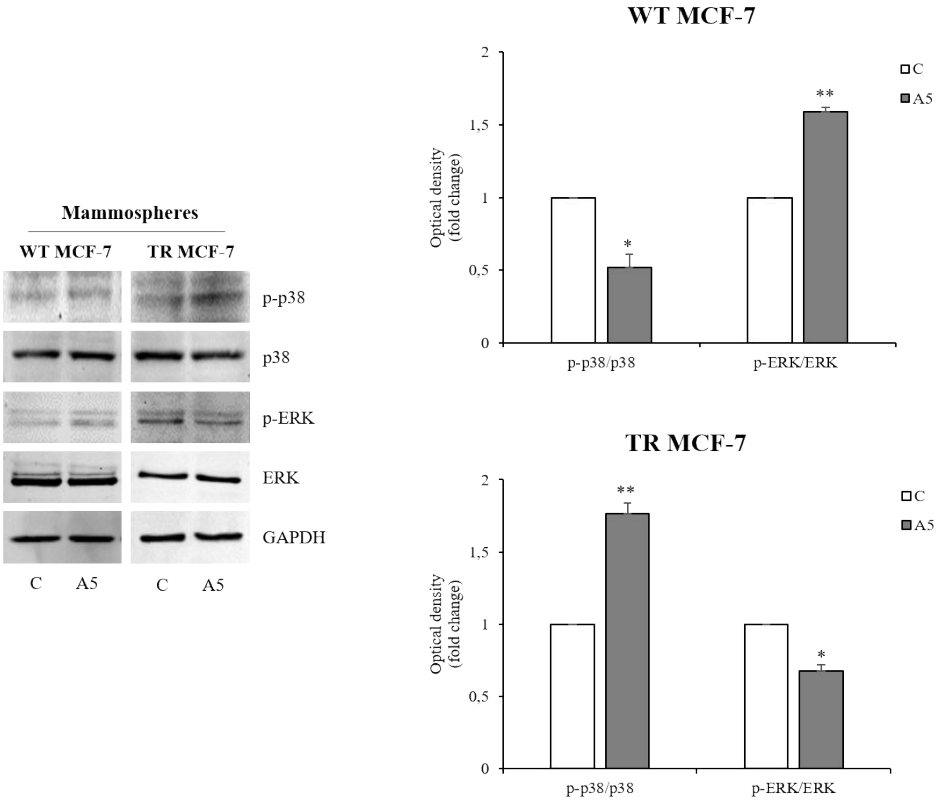


Figure 26. Adiponectin influences quiescent phenotype in TR MCF-7 mammospheres. Levels of phosphorylated (p) p38, and MAPK (ERK1/2Thr44/Tyr42) and total non-phosphorylated proteins were evaluated by Western Blotting. GAPDH was used as a loading control. The histograms represent the means ± S.D of three different experiments in which band intensities, corresponding to the ratio between phosphorylated and non-phosphorylated proteins (p-p38/p38 and p-ERK/ERK), were evaluated in terms of optical density arbitrary units and expressed as fold change of the C assumed as 1. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C; **p<0.01 *vs* C.

The inhibition of CSCs motility is crucial for maintaining the quiescent cell within its niche[196]. Thus, wound healing assay was performed to evaluate the adiponectin effect on the migratory behaviour of WT and TR MCF-7 cells grown as both monolayer or mammospheres culture. Our results demonstrated that already after 24h, TR MCF-7 adherent cells exhibited a greater migration capability than WT

MCF-7 cells upon adiponectin exposure. This effect was even more pronounced after 48h. Contrariwise, adiponectin impaired motility in mammosphere-derived TR MCF-7 cells (Figure 27).

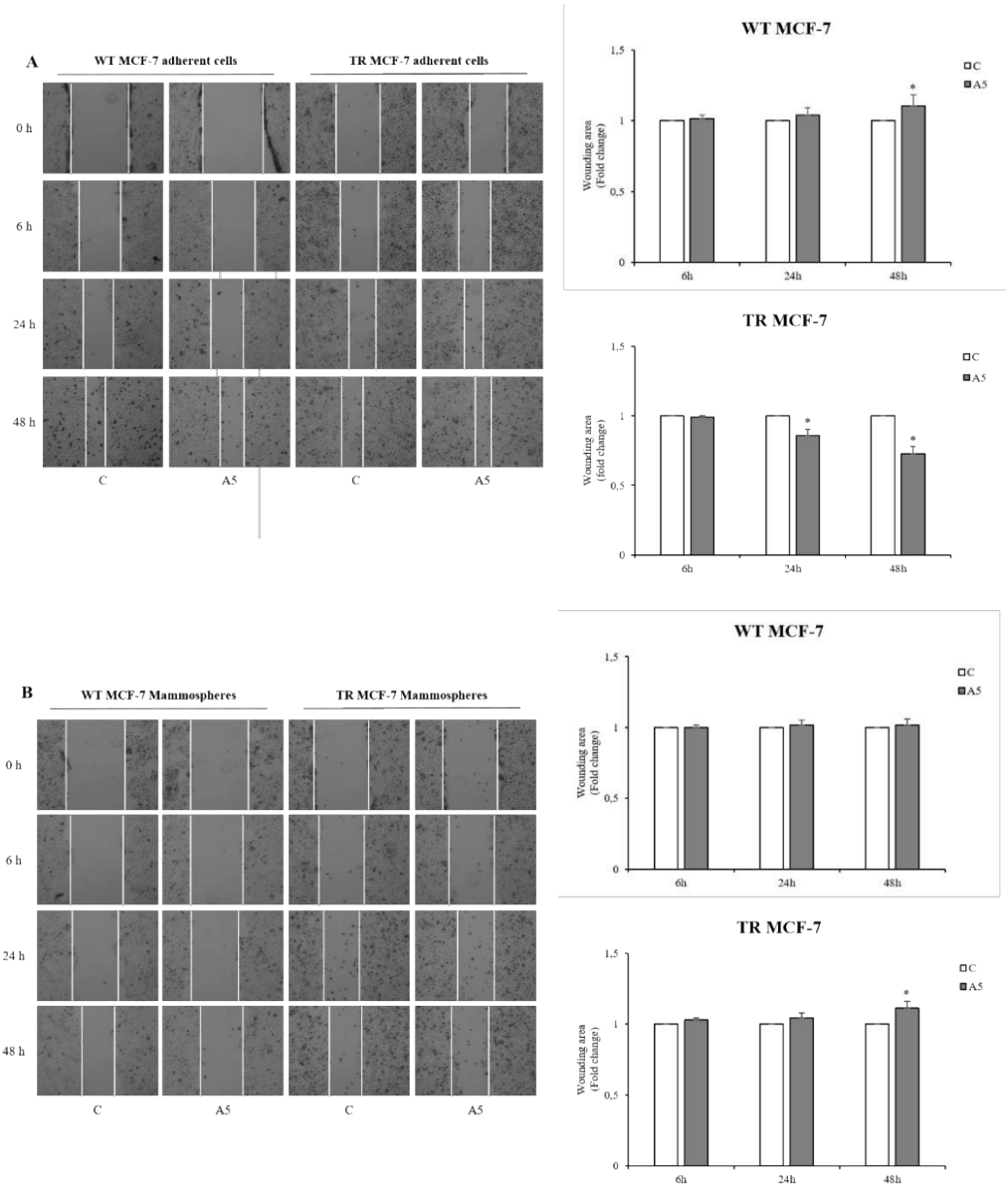


Figure 27. Effect of adiponectin on cell motility in adherent and mammosphere culture. Representative images from *in vitro* scratch wound healing assays in adherent (**A**) and secondary generation mammosphere-derived (**B**) WT and TR MCF-7 cells untreated (control, C) or treated with 5µg/ml (A5) (4x magnification). The white lines show the extent of scratch closure obtained under control condition compared to those with adiponectin-treatment (left panel). The histograms, illustrating wound closure rates at indicated time points during the scratch wound assay (6h, 24h and 48h), represent the means ± S.D. of three different experiments each performed in triplicate. Data were analyzed by Student's T test using the GraphPad Prism 7 software program. *p<0.05 *vs* C.

Thus, adiponectin may maintain hormone-resistant BCSCs in a quiescent state also inhibiting cell motility.

These preliminary results on quiescence in TR MCF-7 mammospheres, treated with low concentration of adiponectin, are extremely intriguing and prospectively predictive for a major frequency of tumor relapse.

Discussion

Several reports have evaluated the role of adipokines in breast cancer progression and recurrence, highlighting a pivotal role of adiponectin in obesity-related breast cancer[36]. Indeed, the pathogenesis of mammary tumor is strongly influenced by the action of the breast microenvironment components which, interacting with each other and with malignant cells, generate a cancer niche that influences the phenotype of the neoplastic cells and sustains drug resistance, cancer recurrence and dissemination[197].

Particularly, adiponectin, the most abundant fat-derived hormone, whose levels are strongly reduced in obesity condition, is well recognized as a crucial regulator of cell proliferation in breast cancer[14,44,198].

Although it impairs cell growth and proliferation in ERα-negative breast cancer cell lines, adiponectin exerts opposite effects in ERα-positive breast cancer cells. Indeed, it has been demonstrated that, through MAPK activation, concomitant with the inhibition of AMPK phosphorylation, adiponectin is responsible for ERα phosphorylation at ser118 and its nuclear transactivation, resulting in cell growth induction. Thus, adiponectin is able to promote ligand-independent activation of ERα, stimulating transcription of receptor-responsive genes involved in cell growth[106]. In addition, adiponectin can influence breast tumor growth through the opposite regulation of cyclin D1 expression, which results up-regulated in ERα-positive cells and down-regulated in ERα-negative cells[105].

Overall, these data evidenced that adiponectin exerts a dichotomic effect on breast cancer growth in relationship to ERα status[105-107].

It is worth noting that tumor mass is recognized as a heterogeneous population of cells in which it is possible to detect a small subpopulation of undifferentiated cells, named as Cancer Stem Cells (CSCs) or cancer-initiating cells, characterized by the capability of self-renewal and to differentiate into various cancer cells. These are responsible to maintain tumor, its malignant behaviour and to initiate metastatic process[199]. The complex interaction between autocrine signals derived from tumor cells and paracrine signals arising in the tumor-associated stroma has been shown crucial in inducing and maintaining a stem-like state in cancer cells[200]. Specifically, regarding breast cancer, it has been evidenced that the components of the tumor microenvironment, encompassing adipocytes, mesenchymal stem cells, tumor associated fibroblasts, immune cells and endothelial cells, through the secretion of different growth factors and cytokines, may increase the proliferation and survival of BCSCs[201]. This let us to wonder if low adiponectin levels, in addition to inducing proliferative effects, may promote the acquisition of stemness traits in ERα-positive breast cancer cells.

First, the phenotypic characterization indicated that adiponectin exposure is able to increase $CD44^{+}/CD24^{-}$ cell subpopulation enrichment and to slightly enhance the capability of mammosphere formation in secondary generation WT MCF-7 mammospheres.

Interestingly, BCSCs differ from other breast cancer cells also in their capability to resist conventional therapeutic approaches, including radiotherapy, chemotherapeutic agents and endocrine therapies. It is well reported that pharmacological treatments based on the administration of estrogen receptor α antagonists (Tamoxifen or Fulvestrant) or aromatase inhibitors (Anastrazole), allow

to inhibit cell proliferation in ERα-positive breast cancer, favoring tumor suppression. However, in recent years, *in vitro* and *in vivo* research, as well as clinical studies, clearly displayed elevations of BCSCs in tumors with acquired resistance to hormone therapy[202]. For instance, previous findings evidenced that *in vitro* exposure with tamoxifen or fulvestrant for five to nine days and *in vivo* 14 days-treatment with both drugs strongly increased BCSCs percentage, suggesting a critical relationship between BCSCs enrichment and acquisition of hormone resistance[203,204].

Based on this evidence, we compared adiponectin effects on hormone-responsive (WT MCF-7) and hormone-resistant (TR MCF-7) ERα-positive breast cancer cells. Specifically, we demonstrated that in TR MCF-7 cells adiponectin promotes a greater enrichment of $CD44^+/CD24^-/ALDH1^+$ expressing-cells, along with an enhanced ability to mammosphere formation and self-renewal, compared to WT cells. In addition, it has been observed a significant higher expression of several genes strongly associated with a stem-like phenotype in TR MCF-7 mammospheres following treatment with adiponectin. Particularly, the most up-regulated genes were members of the Notch family of transmembrane receptors (Notch1, Notch2, Notch3, Notch4), and transcription factors, such as Oct-4, SMAD4 and members of the SOX family (SOX4 and SOX9), known to be involved in the regulation of stem cells biology. Stemness markers expression and the ability to form mammospheres can also be supported by the activation of EMT, process in which epithelial cells lose their differentiated features and acquire mesenchymal characteristics that confer them migratory potential and resistance to apoptosis[200,205]. Here, we showed that in hormone-resistant condition adiponectin

promoted the expression of mesenchymal markers, including Vimentin, α-SMA and TWIST, and reduced the expression levels of E-cadherin, main epithelial marker. In contrast, mammosphere-derived WT MCF-7 cells exhibited a mixture of epithelial and mesenchymal traits upon adiponectin exposure, suggesting that they are partially reprogrammed towards EMT.

BCSCs can escape the action of conventional drugs through adaptation to several strategies[120]. Noteworthy is their ability to maintain themselves in the G0/G1 phase of cell cycle that allows them to remain in a quiescent state and obtain protection from chemo-radiotherapy damage[174]. Our results evidenced the adiponectin decreased Cyclin D1 expression levels in TR MCF-7 mammospheres, revealing a cell cycle arrest in G0/G1 phase, further confirmed by flow cytometry analysis. In accordance with these data, it has been detected an increase of p21 and p27 content, cyclin-dependent kinases inhibitors, in adiponectin-treated TR MCF-7 cells. These events were consistent with reduced proliferation rate, $ki67^{low}$, diminished cell motility, and decreased pERK/p-p38 ratio, additional mechanistic indicators of cell cycle arrest and cellular quiescence. In contrast, our findings showed that in hormone-responsive breast cancer cells, adiponectin was no longer able to impair cell cycle progression and to promote cell dormancy.

In addition to quiescent state, BCSCs exploit several mechanisms to escape death signals, including imbalance among various Bcl-2 family members, which regulate cell fate by promoting or preventing the release into the cytosol of pro-apoptotic proteins from the mitochondrial intermembrane space[177,206]. Indeed, we have found that adiponectin treatment sustained the expression of anti-apoptotic proteins Bcl-

2 and Survivin and negatively affected the expression levels of Bid, tBid and Bax, recognized as pro-apoptotic molecules, in TR MCF-7 mammospheres.

To obtain a deeper understanding of the mechanisms that drives hormone resistance in ERα-positive breast cancer, we compared the protein expression profile of mammosphere-derived WT and TR MCF-7 cells. Interestingly, proteomic analysis revealed a down-regulation of Bid and tBid protein content in adiponectin-treated TR MCF-7 mammospheres, confirming our previous findings.

A common cell death-inducing signal is represented by DNA damage ROS-dependent. Indeed, although ROS generation is a normal product of mitochondrial respiration, it can cause severe damage to cellular proteins, lipids and DNA, resulting in the activation of a signaling pathway that lead to apoptosis[195].

Our results demonstrated that in TR MCF-7 mammospheres, adiponectin, promoting ROS level reduction, prevented the collapse of mitochondrial membrane potential, which impaired the release of cytochrome C and the caspase-dependent inhibitory cleavage of PARP-1. In addition, the reduced ROS production and PARP-1-mediated cell death was also sustained by the loss of p53.

Overall, in the present study it has been evidenced that adiponectin promotes stemness in TR MCF-7 cells grown as mammospheres, inducing increased levels of mesenchymal markers, apoptosis reduction and low Ki67 expression, all features of a cellular phenotype quite close to quiescent state.

In conclusion, it emerges that in hormone-resistant breast cancer low levels of adiponectin, typical of obese subjects, stimulate the enrichment of CSCs that may contribute to relapse. Thus, careful studies, examining the contribution of CSCs subpopulation and the activation of signaling pathways involved in the survival and

maintenance of CSCs, could lead to design tailored therapies for the treatment of breast cancer, particularly in obese patients.

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