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TITLE:

IN VIVO AND IN VITRO EFFECTS OF ATORVASTATIN ON HER2/NEU TUMOR

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1. HISTORY OF STATINS

Initially, several lipid-lowering drugs such as nicotinic acid, cholestyramine, estrogens, and dextrothyroxine were in clinical use but due to their associated side effects none were considered ideal. The demonstration that the feedback suppression of hepatic cholesterol synthesis by dietary cholesterol is mediated by HMGCoA reductase in the conversion of HMG-CoA to mevalonate, paved way for the later development of HMG-CoA reductase inhibitors
(Fig. 1).

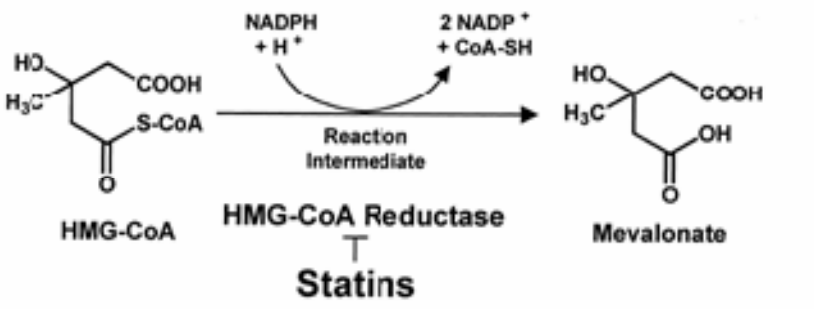
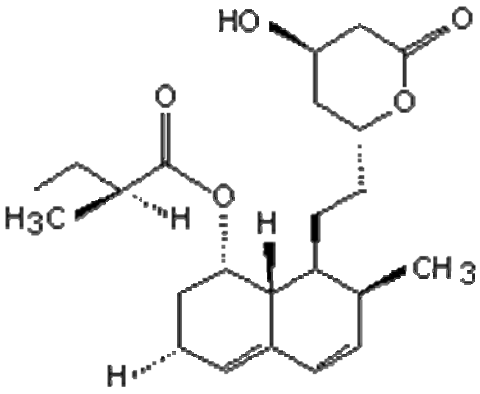


Fig. 1 Structural mechanism for statins inhibition of HMG-CoA reductase

Mevastatin was the first active compound identified in 1971 by Dr Akira Endo and Dr. Masao Kuroda, from Penicillium citrinum to inhibit HMG-CoA reductase (1) and its inactive lactone form can be converted to a water-soluble acid form naturally in the liver or by treatment with an alkali to become an active compound (Fig.2). Mevastatin was found to be ineffective in mice and in rats even at high doses for periods as long as 5 weeks with no reduction in plasma cholesterol. However, its effectiveness came to light when tested in a rat model

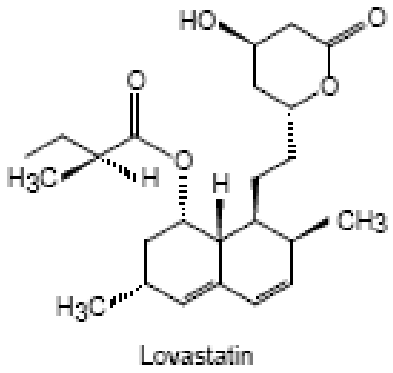
with increased HMG-CoA reductase activity followed by experiments in laying hens and later followed by clinical trials in patients.



Mevastatin

Fig. 2 Structure of Mevastatin

Success with mevastatin encouraged continued search for similar compounds that led to the isolation of lovastatin (Fig.3) (2). Modification of these natural statins led to the development of more potent semi-synthetic and synthetic statins. Simvastatin and pravastatin belong to the seminatural statins while fluvastatin, atorvastatin, cerivastatin, crilvastatin, rosuvastatin and pitavastatin belong to the synthetic statins which differ in their lipophilicity, half life and potency (Table 1)



Lovastatin

Fig. 3 Structure of Lovastatin

Table 1 List of statins

Name of statin	Type	Isolation/modification	Research group/company
Mevastatin (lipophilic)	Natural	*Penicillium citrinum*	Endo et al. [1976]
		Penicillium brevicompactum	Brown et al. [1976]
Lovastatin (lipophilic)	Natural	*Aspergillus terreus*	Alberts et al. [1980]
		Monacus rubber	Endo [1979]
Simvastatin (lipophilic)	Semi-synthetic	A lovastatin analog with an additional methyl group	Hoffman et al. [1986]
Pravastatin (hydrophilic)	Semi-synthetic	A mevastatin analog with an additional hydroxyl group by microbial transformation (*Streptomyces carbophilus*)	Tsujita et al. [1986]
Fluvastatin (lipophilic)	Synthetic	Indolyl derivative	Kathawala [1991]
Atorvastatin (lipophilic)	Synthetic	Substituted H-pyrrole compound	Sliskovic et al. [1991]
Cerivastatin (lipophilic)	Synthetic	Pyridine derivative	Angerbauer et al. [1994] (withdrawn from clinical use in 2001)|
Crilvastatin (lipophilic)	Synthetic	Pyrrolidone derivative	Pan Medica
Rosuvastatin (hydrophilic)	Synthetic	Pyridine derivative	AstraZeneca
Pitavastatin (lipophilic)	Synthetic		Nissan-Kowa

All statins share an HMG-like moiety, which may be present in an inactive lactone form (Fig.4). In vivo, the prodrugs compactin and simvastatin are enzymatically hydrolyzed to their active hydroxy acid forms.

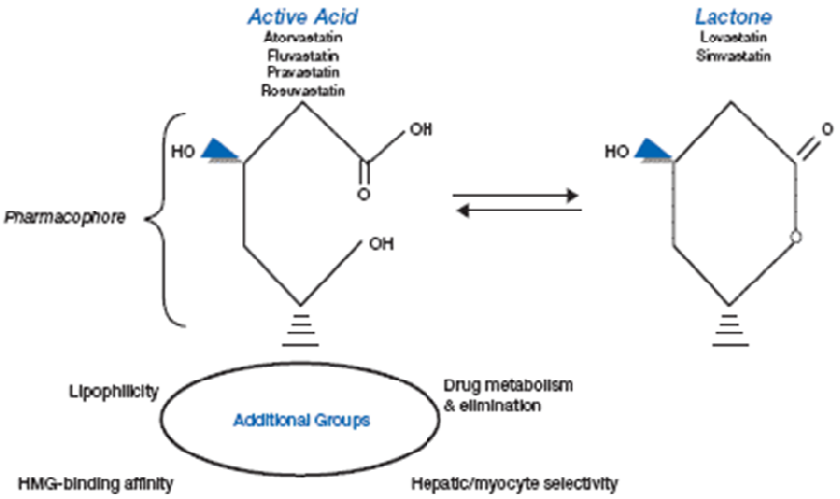


Fig. 4 Hydrolysis of statins

The natural and semi-synthetic statins have a decalin ring structure linked to the HMG-like moiety (Fig.5), whereas the synthetic statins feature fluorophenyl groups that range in character from very hydrophobic (cerivastatin) to partly hydrophobic (rosuvastatin).

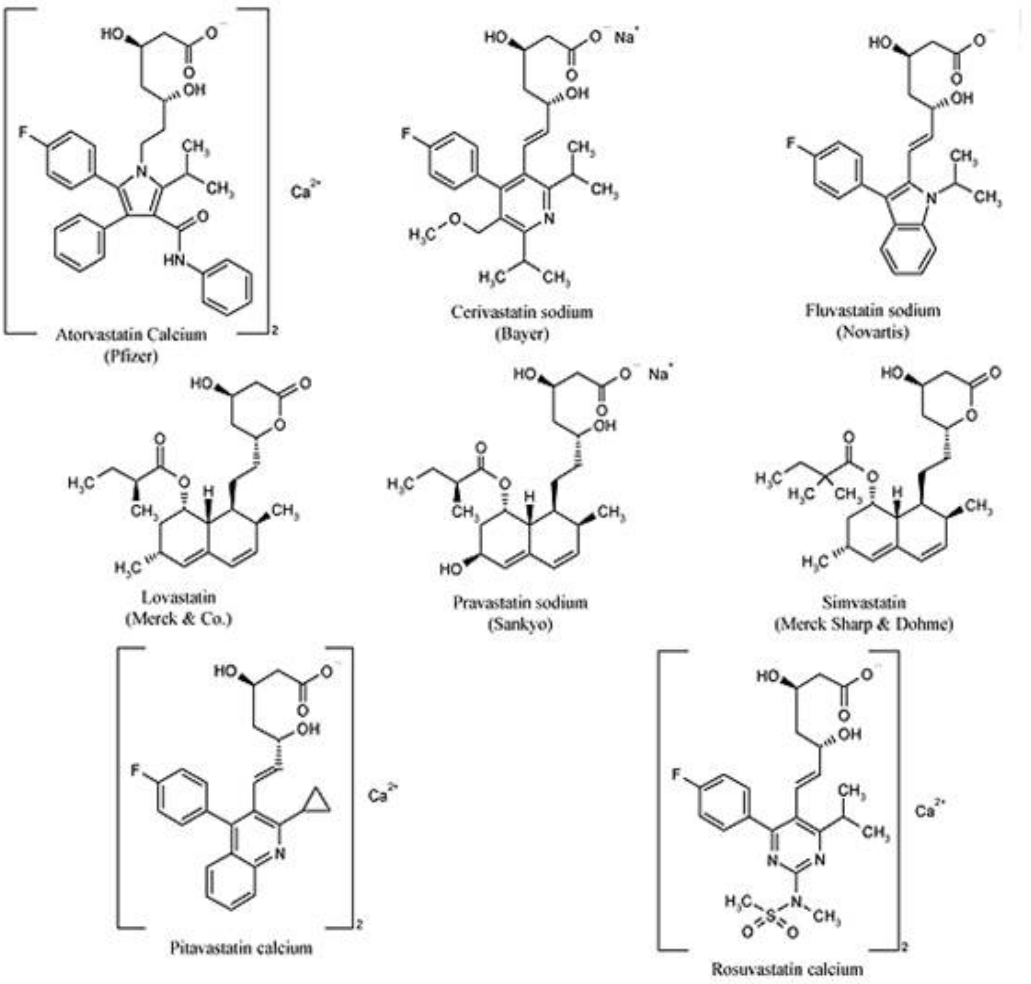


Fig. 5 Structure of statins

CHAPTER 2

2. THE MEVALONATE PATHWAY

Starting from acetil-CoA, the series of reaction that comprises the mevalonate pathway produces farnesil-PP, the precursor for cholesterol, heme A, dicolic, ubiquinone and isoprenilated proteins (3) (Fig 6).

Cholesterol is essential in maintaining cellular membrane structure and integrity, and it also serves as a precursor for the synthesis of steroid hormones and bile acid (4). Dolichol works as a carrier molecule of oligosaccharides in N-linked protein glycosylation for the production of glycoproteins. Ubiquinone is involved in mitochondrial respiration and may also play a significant role in the inhibition of lipid peroxidation (5).

Statins inhibiting the cholesterol synthesis also block the production of geranylgeranylpyrophosphate (GGPP) synthesized from FPP. Both FPP and GGPP are essential for post-translational modification of cellular proteins. In this activation step, the farnesyl or geranylgeranyl moieties are coupled to the protein, resulting in a farnesylated or geranylgeranylated protein. These reactions are catalysed by farnesyltransferase and geranylgeranyltransferase, respectively. This type of protein activation is referred to as (iso) prenylation. Ras, nuclear lamins, and many small GTP-binding proteins such as members of the Rab, Rac, and Rho families to be active, they must first undergo prenylation (*e.g.*, farynesylation or geranylgeranylation) and then they can associate with the plasma membrane (6)**.**

This association is achieved by the addition of a farnesyl moiety (*e.g.*, Ras) or a geranylgeranyl moiety (*e.g.*, Rho) to the COOH terminus of the proteins (7).

Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products and, thus, may have significant influences on many critical cellular functions.

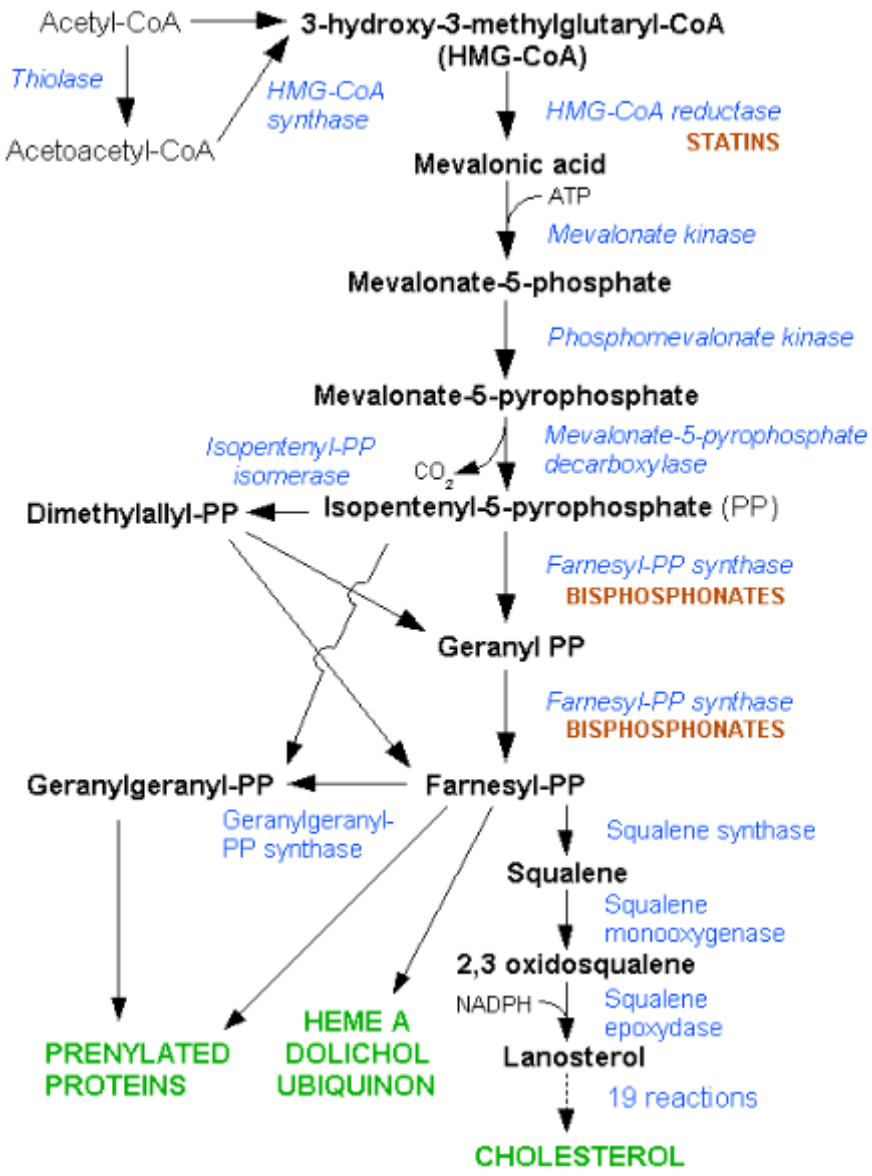


Fig. 6 The Mevalonate pathway

CHAPTER 3

3. MECHANISM OF ACTION

Statins act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the HMG-CoA reductase pathway. Because statins are similar to HMG-CoA on a molecular level they take the place of HMG-CoA in the enzyme and reduce the rate by which it is able to produce mevalonate, the next molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds. This ultimately reduces cholesterol via several mechanisms.

3.1 Inhibiting cholesterol and lipids sintesis

Statins reduce serum cholesterol levels by competitively inhibiting, in the nano Molar range, HMG-CoA reductase (Fig.7). This competitive inhibition results in a failure to catalyze the conversion of HMG-CoA to L-mevalonate, which in turn prevents the downstream biosynthesis of cholesterol. Not only cholesterol synthesis is reduced, but also the low intracellular-cholesterol concentrations activate sterol-responsive-element-binding proteins, leading to transcription enhancement for the gene encoding the low-density lipoprotein (LDL) receptor and subsequent expression of the receptor at the cell surface (8). As LDL-receptor-mediated uptake of LDL cholesterol by the liver is a key factor in determining cholesterol circulating levels, this further reduces LDL cholesterol in blood. The reduction of the level of circulating cholesterol is a primary clinical objective when treating hypercholesterolemia, as it removes one of the main risk factors for atherosclerosis.

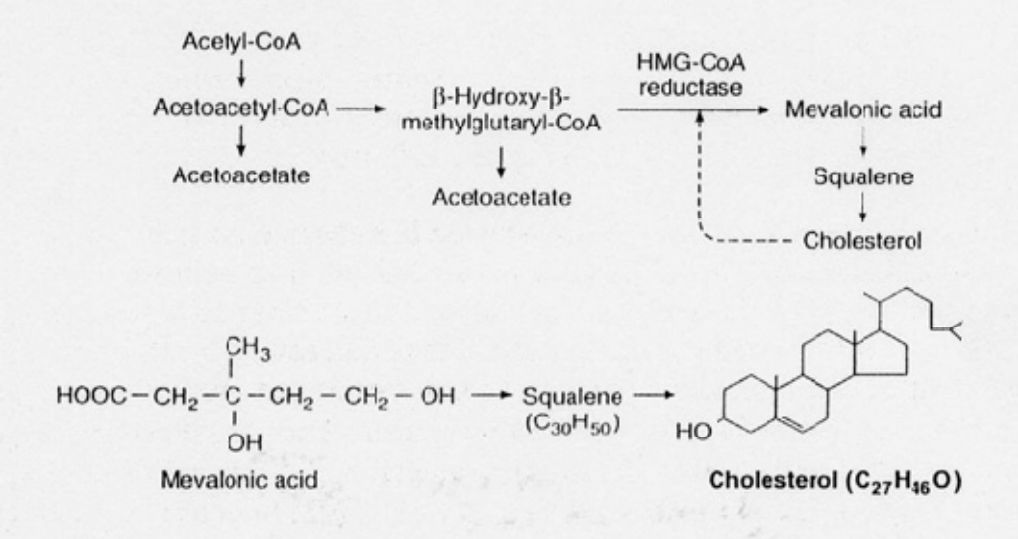


Fig. 7 Cholesterol synthesis

3.2 Statins inhibit protein prenilation

Inhibiting the biosyntesis of mevalonate, statins also inhibit the formation of downstream lipid isoprenoid intermediate such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fig.8). The isoprenoids are lipid moieties that are added to various proteins, including G-proteins and the G-protein subunits RAS, RHO, RAB, RAC and RAP, during post-translational modification (prenylation) and anchor these proteins to the cell membrane (7, 4) (Fig.9). Isoprenoids suppress HMG-CoA reductase by post-translational downregulation. In normal cells, the reductase is subject to complex feedback regulation at the transcriptional, translational and post-translational levels by both sterol and non-sterol products of the mevalonate pathway. Tumour cells, however, are resistant to sterol-mediated feedback and are more sensitive than normal cells to isoprenoid-mediated suppression (9). Post-translational

prenylation by FPP or GGPP is essential for G-protein function. FPP prenylates RAS (farnesylation), which was perhaps the most important target of interest in the early study of statin effects on carcinogenesis. The most relevant studies to date, however, indicate that GGPP prenylation (geranylgeranylation) of other proteins, including the Rho proteins, is the crucial step in the apoptotic, angiogenic and inflammatory effects of statins, as well as other important cellular effects of statins (9). Addition of GGPP or mevalonate generally reverses statin effects, whereas aFPP generally either does not reverse the effects or does so to a lesser degree. The FPP add-back experiments pinpoint the primary influence of geranylgeranylation with respect to farnesylation on statin effects. Even though FPP is the immediate precursor of GGPP, adding FPP generally does not restore GGPP (and therefore reverse statin activity) sincesuch restoration requires isopentenyl pyrophosphate (IPP). Statins block IPP formation upstream of FPP, so IPP is not available for converting FPP into GGPP. Adding mevalonate can reverse the effects of statins because mevalonate can restore IPP for the downstream conversion of FPP into GGPP (10)

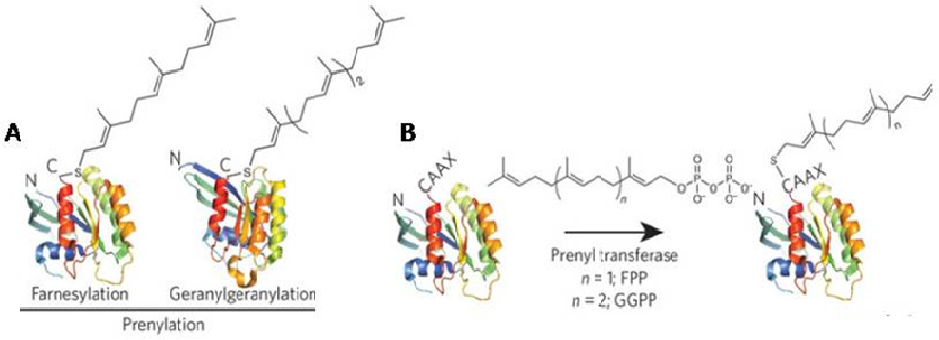


Fig. 8 Protein prenilation. (A)Protein prenilation involves addition of farnesyl or geranylgeranyl moiety to a CAAX motif at the C termini proteins. (B) A set of prenyl transferases includes FTase, GGTase-1 and GGTase-2 transfers farnesyl

(FPP) and geranylgeranyl (GGPP) pyrophosphate precursor to proteins at specific sites.

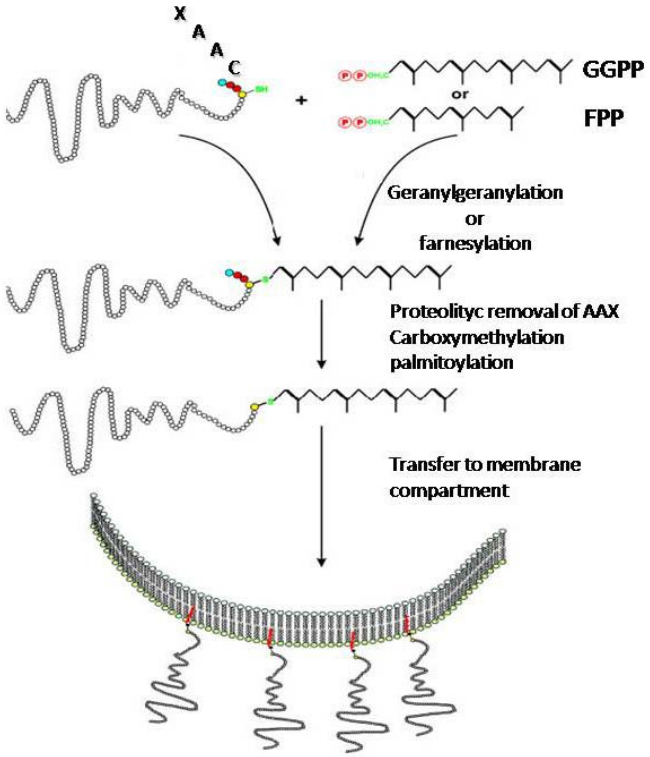


Fig. 9 Protein isoprenylation and post-prenylation reaction.

3.3 Mechanism involving intracellular pathway

3.3.1 MAPK pathway

Statins regulate different signaling pathways through the modulation of enzymes such as Mitogen Activated Protein (MAP) kinases MAP kinases are the family of kinases that transduce signals from the cell membrane to the nucleus.

The MAPK signaling pathways modulate gene expression, mitosis, proliferation, motility, metabolism, and programmed death in response to a wide range of stimuli, including stress. They are serine/threonine kinases that, upon stimulation, phosphorylate their specific substrates at serine and/or threonine residues. Such phosphorylation events can either positively or negatively regulate substrate, and thus the entire signaling cascade activity.

MAPKs consist of three family members: the extracellular signal-regulated kinase (ERK); the c-Jun NH2-terminal kinase (JNK); and the p38- MAPK (11).

An additional MAPK, termed ERK5, has been cloned, and ERK5 is a member of a larger MAPK family that also includes ERK7 and ERK8. ERK1 and ERK2 are well-characterized MAPKs activated in response to growth stimuli. Both JNKs and p38-MAPK are simultaneously activated in response to a variety of cellular and environmental stresses such as changes in osmolarity or metabolism, DNA damage, heat shock, ischemia, inflammatory cytokines, shear stress, UV irradiation ceramide, or oxidative stress (Fig.10).

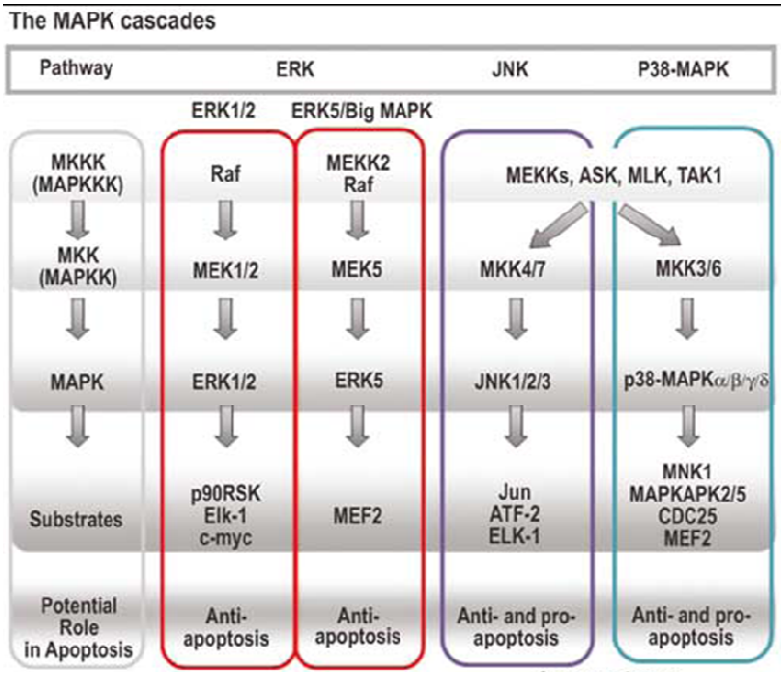


Fig. 10 The MAPK cascades. Three conventional (ERKs, JNKs, and p38-MAPKs) and one additional (ERK5) signaling pathways relay different types of stimuli. Only representative signaling molecules are shown.

- *JNK*

JNKs (also known as stress-activated protein kinases; SAPKs) are ubiquitously expressed, and the JNK stress pathways participate in many different intracellular signaling pathways that control a spectrum of cellular processes, including cell growth, differentiation, transformation, or apoptosis (12). JNK has been reported to activate transcription factors in addition to c-Jun, such as ATF2, Elk-1, p53, and c-My as well as non-transcription factors such as Bcl-2 (12). JNKs are directly activated by the phosphorylation of tyrosine and threonine residues in a reaction that is catalyzed by the dual-specificity kinase MKK4 and MKK7. It has been proposed that JNK activation triggers apoptosis in

response to many types of stress, including UV and γ-irradiation, protein synthesis inhibitors, hyperosmolarity, toxins, heat shock, anticancer drugs , T-cell receptor stimulation, peroxide, or inflammatory cytokines such as TNFa (13) (14) (15)

- *ERK1 and ERK2*

ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in substrates binding. Both are ubiquitously expressed, although their relative abundance in tissues is variable.

ERK signaling cascade are activated by the phosphorylation of a threonine and a tyrosine residue by the dual-specificity MAPK kinases MEK1 and MEK2, which relay Ras and Raf signal transduction to ERK1 and ERK2. (16) (17). ERK1/2 are activated by various growth factors and induce transition from the quiescent state into the cell cycle. ERK signalling pathways are also involved in cell proliferation, differentiation, actin cytoskeleton reorganization, and cell migration. Moreover, ERKs are also involved in stress response and cell death.

- *P-38-MAPK*

The p38-MAPKs were initially termed as mammalian homologues to the yeast protein Hog1 that works as sensor for osmolarity changes (18). In most cases, p38-MAPKs are simultaneously activated with JNKs. At least four isoforms of p38- MAPK have been known: p38-MAPK a, b, g, and d (19). p38-MAPKs are phosphorylated and activated by dual kinases MKK3 and MKK6 at threonine and tyrosine regions. Rho family GTPases take part in the regulation of p38-MAPK

pathway as upstream molecules (20) and activate p38- MAPKs through PAK1-MKK3/6 .

p38-MAPK signaling promotes cell death, whereas it has also been shown that p38-MAPK cascades enhance survival , cell growth, and differentiation (21). In conclusion statins trigger apoptosis and cells proliferation by regulating several signaling, including MAPKs pathway regulation by inhibition of the Ras/Raf/ MEK/ ERK pathway (22).

3.3.2 PI3K pathway

PI3K belongs to large family of PI3K-related kinases or PIKK. Other members of the family included mTOR (mammalian target of rapamycin), ATM (ataxiatelaniecatasia mutated), ATR (ATM and RAD3 related) DNA-PK (DNA–dependent protein kinasae). All possess the characteristic PI3K-homologous kinase domain and a highly conserved carboxyl-terminal tail (23).

The PI3K family (Table 2) comprises eight members divided into three classes according to their sequence homology and substrate preference. All mammalian cells express representatives of the three groups, and the first group was isolated in 1990 (24).

Table 2. PI3K family members

Class	Catalitic subunit	Regulatory subunit	Activation	Products
Ia	p110α p110β p110δ	p85	RTK, RAS	PtdIns-3,4,5-P_3 PtdIns-3,4-P_2 PtdIns-3-P
Ib	P110γ	p101	Heterotrimeric G proteins	PtdIns-3,4,5-P_3 PtdIns-3,4-P_2 PtdIns-3-P
II	PI3KC2α PI3KC2β PI3KC2γ		RTK, integrins	PtdIns-3,4,-P_2 PtdIns-3-P
III	VSP34p			PtdIns-3-P

There is mounting evidence that phosphoinositide 3'-kinase (PI3K) is involved in statin-induced effects on proliferation and induction of apoptosis. Downstream targets of PI3K are protein kinase B (PKB or Akt) and $p70^{S6K}$. The PI3K/Akt pathway promotes survival by affecting the Bcl-2 protein family. Activated Akt suppresses apoptosis by phosphorylation of the proapoptotic protein BAD, disabling heterodimerisation of BAD with antiapoptotic proteins Bcl-2 and Bcl-Xl (25) (26) (Fig. 11)

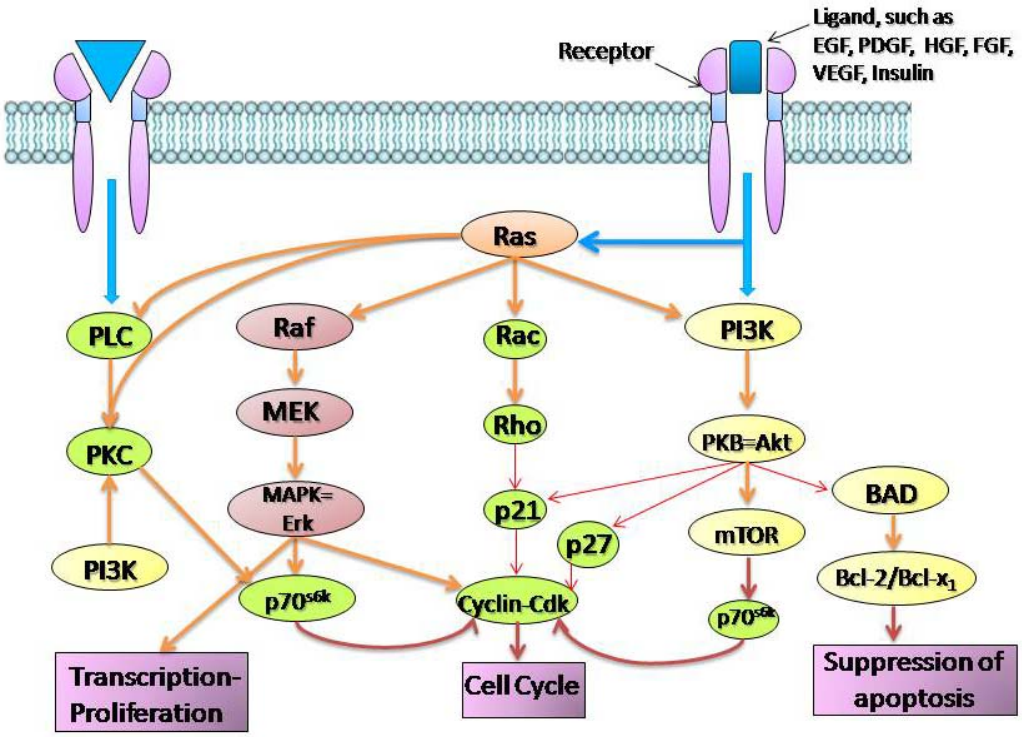


Fig. 11 Intracellular signal transduction cascades.

Many work show that the effects of statins on signaling cascade, such as MAPK and PI3K, are ambiguous. This can be explained by two observation. Nguyen et al. (27) supports that the requirement of specific signaling pathway for malignant transformation is dependent on the type of cells and the nature of the oncogenes.

Moreover, regulation of the MAPK and PI3K pathway is rather complex. It was shown that these pathway crosstalk at the level of Raf-1 and Akt. The PI3K signaling cascade has a role in regulation of the Erk pathway, both positively and negatively. This regulation depends on the nature and strength of the incoming signal (28). These findings indicate that activation of signaling pathway varies in different malignant cell types. This may explain the unambiguous effects of statins on these pathway.

3.4 Statins arrest growth and cell cycle

In vitro studies on various cell lines have shown that statins have growth inhibitory potentials, either by induction of G1-arrest, G2/M-arrest or cell death (29) (30). Since chromatin condensation and DNA laddering have been observed (31) (32), statins induced cells death is considered to occur via apoptosis. These phenomena are both time- and dose- dependent.

Indeed cerivastatin induce G1-arrest in breast cancer cells, but signs of apoptosis were not observed (33), this data suggest that statin- induced effects on proliferation and apoptosis are either independent (34) or dependent on the statin concentration. Other works in the literature show that growth arrest and apoptosis following lovastatin treatment in concentration ranging from 0,1 to 100 mM depending of cells line type (35) however lovastatin monotherapy may not be sufficient to inhibit proliferation and induce apoptosis. Wong et al. (36) showed that different statins were not equipotent in inducing apoptosis , for instant in acute myeloid leukemic (AML) cell line, cerivastatin was at least 10 time more potent than other statins in the induction of apoptosis.

Statins can be divided into three group with respect to their anti- proliferative properties; the inhibitory potency of simvastatin. Lovastatin, fluvastatin, and

atorvastatin is in the same order of magnitude, whereas pravastatin is significant less potent and cerivastatin is more potent (37).

CHAPTER 4

4. STATINS AND CANCER

Numerous studies on statin and various cancers have been carried out by different research group.

HMG-CoA reductase by preventig the activation of Ras/Rho proteins, exert pleiotrophic benefits on the cardiovascular apparatus, as well as on colon, lung, kidney, bone, central nervous system disease; for the same reason statins may be considered as potential adjuvant drugs in anticancer therapy. In different experimental models statins have reduced the tumor cell proliferation and enhanced the cytotoxic efficacy of conventional chemotherapeutic drugs. However few and conflicting data exist about the efficacy of statins in preventing tumor onset in animal models and their efficacy in clinical trials.

4.1 Statins and Breast cancer

There is extensive evidence that different statins suppress the growth and/or induce apoptosis of breast adenocarcinoma cells *in vitro*. Such effects have been demonstrated in response to various statins, including fluvastatin, simvastatin, lovastatin and pravastatin (38) (39) (40). The antitumor effects of statins in breast carcinoma cells have been associated with suppression of the MEK/Erk pathway. In addition, some tumors elevation of protein levels of the tumor suppressor, p21, occurred in a statin-dependent manner (40). Other studies have suggested that simvastatin induces apoptosis of breast cancer cells via engagement of the JNK pathway, and in a manner independent of their estrogen receptor or p53 expression status (41). Thus, dual regulation of Map kinase pathways in breast cancer cells, involving both suppression of Mek/Erk

activity and induction of JNK activity, is associated with the antitumor effects of statins. Recent studies have demonstrated important roles for nitric oxide and arginase-dependent pathways in the generation of statin-inducible pro-apoptotic effects in MCF-7 breast carcinoma cells (42). So far, epidemiological studies have not shown any association between statin use and a decrease in the incidence of breast cancer (43), but there has been some evidence indicating a reverse association between post-diagnosis lipophilic statin use and risk of breast cancer recurrence (44). Indeed it was demonstrated that there is not a evidence of a protective effect of statin against breast cancer, Indeed the possibility that statins have an effect depends on the type of stain used and the type of cancer, in fact among the cases considered in women suffering from invasive cancer treatment with statins had no effect (45)

4.2 Statins and Colorectal cancer

Statins have been shown to exhibit antitumor effects against various solid tumor cell lines. Several studies have established that different statins suppress growth and/or induce apoptosis of colorectal carcinoma cell lines (46) (47) (48) (49). Interestingly, HMG-CoA reductase activity and LDL receptor levels were detected at higher levels in colonic cancer cells than in normal mucosa (48), suggesting a potential involvement of the cholesterol pathway in malignant transformation of colon cells (48). Importantly, it has been shown that mevastatin induces strong upregulation of p21CIP1/WAF1 protein to mediate its growth inhibitory effects on colon cancer cells (45). The extensive preclinical evidence indicating that statins inhibit the growth of colon carcinoma cells has led to efforts to determine whether statins can be used as preventive agents for colorectal cancer. *In vivo* studies in mice have shown that atorvastatin inhibits

intestinal tumorigenesis and that when given in combination with celecoxib, it enhances its chemopreventive efficacy in an APC^{min} mice (50). Moreover, recent epidemiological studies in humans have shown that the use of statins is associated with significant reduction in the risk of colorectal cancer after adjustment for other known risk factors (51). Another recent study in which statins were used regularly for three months did not demonstrate a statistically significant decrease in the overall risk for colorectal cancer (52). However, the use of statins was associated with a substantial decrease in the risk of cancer (53), further suggesting that statins have antitumor effects against colorectal cancer cells *in vivo.* Additional epidemiological studies will be required to definitively determine whether statins have roles as chemopreventive agents for colorectal cancer. In addition, the potential use of statins as components of chemotherapy regimens for the treatment of advanced colorectal cancer, or in the adjuvant setting, deserves evaluation in future clinical trials.

4.3 Statins and Lung cancer

Among the different solid tumors, lung cancer is one on which the effects of statins are very prominent. There is extensive evidence that different statins exhibit potent growth inhibitory and pro-apoptotic effects both on non-small cell lung (54), as well as small cell lung carcinoma cell lines (55). In addition, lovastatin can suppress the formation of tobacco-specific nitrosamine, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumors in mice at an early promotional stage (56). Interestingly, the effects of statins on certain type of lung carcinoma cells may relate to their ability to inhibit the function of the epidermal growth factor receptor (EGFR) (54), which is expressed at high levels in certain types of lung cancer. Previous studies have

shown that lovastatin-treatment inhibits EGF-induced EGFR autophosphorylation, and that combining lovastatin and gefitinib results in enhanced inhibition of AKT activation by EGF and enhanced cytotoxicity (54) .

Based on the extensive in vitro and in vivo evidence on the effects of statins on lung cancer cells, a retrospective case-control study evaluated the association of lung cancer and the use of statins in patients enrolled in the Veterans Affairs (VA) Healts Care System in the United States were taken into account around 2000 lung cancer cases among statins users and in that was found a 45% reduction in lung cancer risk among statins users compared to non users (57). It important to note that the effects of statins on lung carcinogenesis, appear to be independent of factors such as diabetes, use of alcohol or smoke. Also in this study was shown an increase in protection from lung cancer in people making use of statins for more than 6 months; the authors hypothesis is that increasing duration of statin use was associated with a protective effect on lung cancer risk.

Such highly encouraging initial results suggest that statins may have clinical potential as chemopreventive agents in lung cancer.

4.4 Statins and Prostate cancer

It has been previously demonstrated that prostate cancer cell lines contain more lipid rafts than their normal counterparts and are more sensitive to cholesterol-depletion induced cell death using MBCD (58). Such increased expression of lipid rafts is similar to what has been also observed in breast cancer cell lines (58) . Lovastatin, fluvastatin, and simvastatin have been all shown to inhibit proliferation of the PC-3 and LNCaP prostate carcinoma cell lines (59) and such inhibition of cell growth correlated with induction of cell

cycle arrest at the G1 phase (59). Consistent with this, another study has demonstrated that low dose lovastatin induces senescence and G1 cell cycle arrest in human prostate cancer cells (60). Interestingly, overexpression of a constitutively active form of RhoA was found to reverse lovastatin-induced senescence, suggesting that the induction of senescence by lovastatin (60) may be mediated, at least in part, by its effects on RhoA. In studies in which the role of lipid raft cholesterol content on *in vivo* cell survival mechanisms was examined, a correlation was established between cholesterol levels and protein tyrosine phosphorylation in lipid rafts isolated from LNCaP/sHB xenograft tumors in SCID mice (61). Cholesterol elevation also promoted tumor growth, increased phosphorylation of Akt, and reduced apoptosis in the xenografts (61), further emphasizing the role of cholesterol lipid rafts in prostate cancer cells. In the same study, it was demonstrated that by lowering raft cholesterol content, simvastatin decreased Akt activation and promoted apoptosis in prostate cancer cells (61) . It should be noted that beyond the effects of statins on RhoA (60) (61) , a major regulator of prostate cancer cell growth (62), there is evidence that statins induce p53-independent transcriptional regulation of p21WAF1/CIP1 in human prostate carcinoma cells (63).

There have been some epidemiological studies aimed to examine the potential roles of statins as chemopreventive agents in prostate cancer. Remarkably, treatment with statins was found to decrease serum levels of prostate specific antigen (PSA) in men treated for hypercholesterolemia, as compared to normal controls (64). Another study, in which the effects of statins were analyzed in a large prospective cohort study of 34,989 US male health care professionals, it was found that the use of statins was not associated with the overall risk of prostate (65). However, there was an association between statins use and reduced risk of metastatic prostate cancer (65), further suggesting that statins exhibit clinical activity against prostate cancer. Other studies have shown that

statins, especially atorvastatin, may improve clinical presentation and biochemical progression-free survival after brachytherapy (66). It should be also noted that beyond their direct antitumor effects, statins might have additional beneficial effects in patients treated for prostate cancer. It is well known that a major complication of surgical treatment for patients with prostate cancer is erective dysfunction. A recent study demonstrated that treatment of prostate cancer patients with atorvastatin results in earlier recovery of erective function after nerve sparing prostatectomy (67). In general, there is a plethora of evidence indicating a potential useful role for statins in the management of prostate cancer. Further studies to definitively address the relevance of statins in the prevention or treatment of prostate cancer are warranted and may result in the development of novel future approaches for the treatment of this malignancy.

CHAPTER 5

5. CANCER INITIATING CELLS

The model of carcinogenesis, postulated by Nowell and Vogelstein, describes the formation of a tumor by the sequential accumulation of mutations in oncogenes and tumor suppressor genes. In this model, tumors are thought to consist of a heterogeneous population of cells that continue to acquire new mutations, resulting in a highly dynamic process, with clones that out compete others due to increased proliferative or survival capacity. However, continued research on stem cells have suggested a new theory on the complexity in the process of malignant transformation and preservation. Furthermore, it has recently become clear that the bulk of cells that make up a tumor (be that primary tumors or metastases) are derived from a small subpopulation of cancer initiating cells (CICs). CICs are distinguished from the bulk population of tumor cells by their ability to successfully seed new tumors when implanted in low numbers into experimental animals. In contrast, the non-CIC population cannot initiate tumor growth in vivo even when implanted in high numbers (68). Much of the evidence supporting the notion of CICs has been derived from the analysis of haemapoietic tumors, but recent findings indicate that similar principles also hold true in solid tumors, including breast cancer (68). CICs share properties with stem cells in normal tissues, dividing both to self-renew and to give rise to progeny that make up the bulk of the tumor mass (Fig 12). While CICs may be directly derived from transformation of normal stem cells, there is also evidence that CICs can be derived by transformation of transit amplifying cells, for example in the context of breast tumors [*e.g.* (69)]. Evidence is accruing that common signaling pathways such as the Wingless, notch, hedgehog and Bone Morphogenetic Proteins (BMP) pathways regulate the properties of both normal stem cells and CICs [(70) (71) (72) (73) (74)]. The

ability of CIC progeny to differentiate into tumor cells with different phenotypes may account for the heterogeneity in tumor cell populations.

Furthermore, there is now strong evidence that primary tumors can remotely induce the survival and growth of disseminated tumor cells within the organs to which they will metastasize. Integration of these new insights with previous ideas about how metastasis works should allow major advances to be made in our understanding of the process of tumor dissemination.

The mechanisms leading to the metastatic dissemination of tumor cells appear to be similar for many different types of cancer, including breast tumors, and are associated with multiple cellular processes. These include invasion into surrounding tissue, intravasation into blood or lymphatic vessels, survival and dissemination through the blood or lymphatic circulation, colonization of distant organs by adhesion to the vessel wall, extravasation and invasion into distant organ parenchyma, and finally metastatic outgrowth in the distant organ (75). Thus, metastasis is a highly complex problem with many facets. Several hypotheses have been developed over recent years to explain how this process, or aspects of it, is regulated.

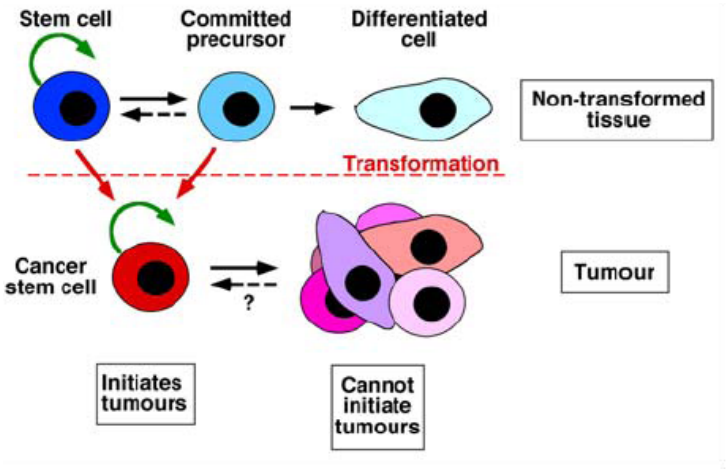


Fig. 12 Schematic representation of relationship between normal stem cell, transformed cells and cancer stem cells.

5.1 Mammary Stem Cells

The adult mammary gland is composed of at least three cell lineages including myoepithelial cells, ductal epithelial cells, and alveolar epithelial cells. These cell types form the basal layer of ducts and alveoli, line the lumen of the ducts, and synthesize milk proteins respectively. Mammary gland development and remodeling during puberty, pregnancy, lactation and involution is complex and dynamic. These processes include rapid cell proliferation, differentiation and apoptosis. The first evidence supporting the existence of mammary stem cells came in the 1959 when it was demonstrated that the transplantation of portions of mammary tissue from donor mice into the epithelium –free recipient mice led to functional mammary outgrowth containing ductal, alveolar and myoepithelial cells (76).

The further characterization of mammary stem cells was aided by the development of an in vitro propagation system for stem cells in suspension culture. In analogy with the in vitro propagation system for neuronal stem cells, which form neurospheres in suspension (77), mammary stem cells form mammospheres in suspension culture. These mammospheres are spherical cell structures that are able to survive and proliferate in the absence of attachment to an exogenous substratum. As is the case for neurospheres, the mammospheres are highly enriched in bi- or multipotent cells, as demonstrated by the ability of single cells isolated from mammospheres to generate multilineage colonies when cultured under conditions promoting differentiation. In addition, it was demonstrated that single cells obtained from mammospheres are capable of generating secondary mammospheres, suggesting that these single cells are capable of self-renewal.

To isolate and enrich stem cells from mouse and human mammary glands were used techniques that utilized the biological properties of stem cells to develop enrichment systems. Farther capability to generate sphere, stem cells are mainly quiescent and in the G0 state. They are able to retain bromodeoxyuridine (BrdU) or 3H-thymidime longer than the cycling cells [(78) (79)]. Also, stem cells express specific membrane glycoprotein transporters, which allow their identification by membrane staining. These characteristics have allowed identification of surface markers useful in distinguishing mammary stem cells from bulk of mammary epithelial cells.

5.2 Breast Cancer Stem Cells

The main breast cancer tumors are invasive carcinoma, and 10-15% of breast cancer are invasive lobular carcinomas. Additional rare types constitute less than 5-10% of breast cancers [(80) (81) (82)]. According to their gene

expression signatures, invasive ductal carcinomas can be classified into five subtypes: luminal A, luminal B, ERBB2 (Her2/Neu), basal and normal-like. The luminal subtypes are mostly estrogen receptor (ER)-positive. The low-grade and low-proliferation luminal A tumors are sensitive to hormonal therapy and often have better prognoses and clinical outcomes than the basal and ERBB2-like subtypes. One fundamental question that needs to be addressed is whether different subtypes of breast cancers are derived from different origins, *i.e.* different breast cancer stem cells (83). These different breast cancer stem cells in turn may explain the different response of each breast cancer subtype to the same therapy.

It has been reported that there is a putative stem cell-like population in human breast cancers. These so-called breast cancer stem cells have been isolated from human breast tumors or breast cancer-derived pleural effusions using cell surface markers in combination with flow cytometry; Al-Hajj et al. defined these cells $CD44^{high}/CD24^{low}$. Using NOD/SCID mice, they showed that these cells have higher tumorigenic potential than bulk tumor cells. When, $CD44^{high}/CD24^{low}$ cells were isolated from tumors, these cells could be transplanted to NOD/SCID mice and generate new tumors (84).

Another study used a low attachment *in vitro* culture system to show that single cell suspensions of $CD44^{high}/CD24^{low}$ cells from human breast cancers were able to proliferate extensively and form clonal non-adherent mammospheres. These mammospheres were more tumorigenic than the established breast cancer-derived cell lines including MCF-7 and B3R (85).

There have been many studies adapting these systems to isolate breast cancer stem cells from either breast tumors or breast cancer cell lines for further characterization such as analysis of signal transduction pathways, gene expression, and microRNAs to find factors important for the self-renewal, tumorigenesis, and drug resistance properties of breast cancer stem cells.

5.3 Statins and Cancer Stem Cells

Recent studies on the existence of stem cells in tumor tissues, which are assigned the responsibility of the progression and the formation of recurrent tumors, have opened new avenues for cancer research. Several common properties exist between CICs and embryonic stem cells (hESCs), namely their self-renewal potential, high proliferative capacity, long population doubling time, increased telomerase activity, high nuclear to cytoplasmic ratios and induction of tumors in immune- deficient mice. Additionally, both stem and cancer stem cells share the same signaling mechanisms. For these reasons it thus may be useful to study new compounds that will specifically target and destroy CICs, which once eliminated may prove beneficial against tumor progression and recurrence preferably with minimal or no side effects.

RESULTS

Over expression of the ErbB2 (HER2/Neu) receptor tyrosine kinase has been found in various human malignancies, including breast, ovarian and gastric carcinomas, non-small cell lung cancer, and salivary gland cancers, and has be associated with poor prognosis . Due to this enhanced expression on tumor cells and its involvement in essential signaling processes, ErbB2 constitutes an important target for directed cancer therapy.

In this study we evaluate the potential anticancer effect exerted by Atorvastatin on Her2/neu mammary tumor.

1) In the first part of this study we investigated whether the lipophylic statin, atorvastatin, at a dose-schedule superimposable to that adopted in anticholesterolemic therapy have any effect on the development of breast tumors in BALB-neuT mice and which pathways of the transformed epithelial cells are affected by the statin.

   The results obtained are summarized below and contained in the paper "**The inhibition of NF-kB activity is required for the anti-proliferative effect of atorvastatin in Her2/neu-positive mammary cancer"** submitted for publication and currently under review in the journal " **Breast Cancer Research**".

To analyse the anticancer effect of atorvastatin on Her-2/neu positive breast cancer, we used BALB-neuT mice that are one of the most aggressive model of mammary carcinogenesis. BALB-neuT are BALB/c mice transgenic for the rat (r) HER2 transforming oncogene under the transcriptional control of the MMTV promoter. In 3-week-old BALB-neuT mice, the rHER2 is markedly overexpressed on the surface of the cells of the rudimental mammary gland, at the 6th week, the rHER2+ cells give rise to a widespread atypical hyperplasia of small lobular ducts and lobules. Foci of ''in situ'' carcinoma first apparent around the 15th week, and evolve into invasive lobular carcinomas by the 20th week. Ten weeks later an invasive lobular carcinoma is present in all mammary glands.

First of all we studied the ability of atorvastatin to inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase in a Her-2/neu+ cloned line generated from a BALB-neuT mammary gland carcinoma (TUBO cells).

Atorvastatin significantly reduced the *de novo* synthesis of isoprenoids. As a consequence of the reduced isoprenoids synthesis, the GTP-binding of Ras and RhoA, which is an index of the isoprenylation and activity of monomeric G-porteins, was lower in cells incubated with atorvastatin. Then we examined some downstream signalling pathways of Her2, focusing on MAP kinases ERK1 and ERK2, which are effectors of Ras. ERK1 and ERK2 were constitutively phosphorylated in TUBO cells and such activation was dramatically reduced by atorvastatin.

To evaluate the capability of atorvastatin to reduce the in vivo growth of TUBO cells, BALB/c mice were injected s.c. with a lethal dose of TUBO cells at day 0, when atorvastatin treatment began. Both in controls and in atorvastatin treated mice the tumors grew steadily, and 18 days after TUBO challenge all mice had tumor.

Then, we tested atorvastatin ability to inhibit mammary carcinogenesis in female BALB-neuT mice. In this case the animals were treated starting from 10

weeks of age, when in their mammary glands atypical hyperplasia and in situ carcinomas are present. Atorvastatin administration did not delay tumor progression and all treated mice had at least one palpable tumor by week 25 and at 37 weeks all animals died.

These results show that atorvastatin is an excellent inhibitor of mevalonate pathway and Ras and RhoA signalling, however this is not sufficient to block or at least reduce the occurrence of cancer in the systems we have taken into account. Indeed, by decreasing the levels of mevalonate-derived metabolites, atorvastatin contemporarily increased the activity of NF-kB transcription factor, that acted as a supplementary signal to sustain the tumor growth. Only the simultaneous suppression of Her2/neu-dependent pathways and NF-kB activation allowed atorvastatin to exert an antitumor activity.

The inhibition of NF-kB activity is required for the anti-proliferative effect of atorvastatin in Her2/neu-positive mammary cancer.

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ABSTRACT

Introduction. The widely used anticholesterolemic drugs statins decrease the synthesis of cholesterol and the isoprenylation and activity of small G-proteins like Ras and Rho, whose effectors are often critical for cells proliferation. Thanks to this property, it has been hypothesized that statins may have anti-tumor activities.

Methods. We investigated whether atorvastatin had an anti-proliferative effect on mammary cancer cells from Her2/neu transgenic (BALB-neuT) mice. Experiments were performed in vitro on TUBO cells, an established mammary cell line from BALB-neuT mice, and in vivo. The experimental results were analyzed by ANOVA and Student's t Tests, the survival curves by Chi square Test.

Results. The treatment of TUBO cells with atorvastatin decreased the activity of mevalonate pathway and the Ras and RhoA signaling. Differently from mammary glands of BALB/c mice, the tumor tissue from BALB-neuT animals, which developed cancer with 100% penetrance, had constitutively activated Ras and ERK1-2, that were reduced by the oral administration of atorvastatin. However, the statin did not prevent the tumor growth in mice and did not reduce TUBO cells proliferation. Indeed, by decreasing the levels of mevalonate-derived metabolites, atorvastatin contemporarily increased the activity of NF-kB

transcription factor, that acted as a supplementary signal to sustain the tumor growth. Only the simultaneous suppression of Her2/neu-dependent pathways and NF-kB activation allowed atorvastatin to exert an antitumor activity.

Conclusions. Our study demonstrates that Her2/neu positive mammary cancers have redundant signals to sustain their proliferation and opens the possibility to conceive new anti-tumor approaches based on the association of statins and targeted-therapies.

INTRODUCTION

Statins, which inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) enzyme, are the most effective drugs to reduce the intracellular synthesis of cholesterol and prevent the onset of atherosclerosis and cardiovascular diseases [1]. They also decrease the synthesis of isoprenoid side products of the mevalonate cascade, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), impairing the isoprenylation and activity of small G-proteins, like Ras and Rho. When isoprenylated, monomeric G-proteins bind and hydrolyse GTP, thus activating downstream effectors, which in turn may modulate cell proliferation, cytoskeleton remodeling, motility and angiogenesis [2]. By preventing the activation of Ras/Rho proteins, statins exert pleiotropic benefits on the cardiovascular apparatus, as well as on colon, lung, kidney, bone, central nervous system diseases; for the same reasons they have also been proposed as potential adjuvant drugs in anticancer therapy [3]. In

different experimental models statins have reduced the tumor cell proliferation [4-6] and enhanced the cytotoxic efficacy of conventional chemotherapeutic drugs [7,8].

However, few and conflicting data exist about the efficacy of statins in preventing tumors onset: atorvastatin has not reduced the incidence of methylnitrosurea-induced breast cancers in rats [9] and epidemiologic studies on patients taking HMGCoAR inhibitors showed little or no reduction of the incidence of breast, lung and prostate cancers [10]. On the other hand, restricting the focus on breast cancer, women treated with statins develop tumors with a less aggressive phenotype [11] and display a lower frequency of tumor recurrence [12].

Her2/neu positive breast tumors represent the 20-30% of total breast cancer and are often associated to poor prognosis and to chemo- and radio-resistance [7]. In consequence of the constitutive activation of the Her2 downstream transducers, which include the Ras/Raf/Mitogen-Activated Kinases (MAPKs) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, these tumors are characterized by an higher proliferation rate and a pronounced resistance to apoptosis [7,13]. An active mevalonate pathway seems critical for the growth of Her2 positive tumors: indeed the amounts of plasma membrane cholesterol affect Her2 conformation and activity and the levels of isoprenoid dolichol regulate the Her2 glycosylation. Furthermore, an adequate supply of

FPP is mandatory for the activity of Ras protein and for the subsequent activation of Ras-downstream effectors [7]. Therefore the inhibition of mevalonate pathway by statins has been associated to specific Her2-targeting therapies, in order to achieve a strong anti-proliferative effect [7,8]. No data exist on the effects of statins as single agents applied to Her2 positive breast cancer cells.

BALB/c mice transgenic for the transforming rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus promoter (BALB-neuT mice), develop breast cancer with 100% penetrance [14]. Starting from atypical hyperplasia (at week 3), mammary glands display at 8 weeks multiple in situ carcinomas, which become invasive tumors between weeks 17 and 22 [15].

In this study we investigated whether the lipophylic statin atorvastatin, at a dose-schedule superimposable to that adopted in anticholesterolemic therapy (40 mg/Kg/day,) [16], have any effect on the development of breast tumors in BALB-neuT mice and which pathways of the transformed epithelial cells are affected by the statin.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS), penicillin-streptomycin (PS) and RPMI 1640 were supplied by Sigma Chemical Co (St. Louis, MO), plastic ware for cell

culture was from Falcon (BD Biosciences Discovery Labware, Bedford, MA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules CA), the protein content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit from Sigma Chemical Co. Atorvastatin was purchased from Sequoia Research Products (Pangbourn, UK). FTI-I and GGTI-286 were acquired from Calbiochem (San Diego, CA). When not otherwise specified, all the other reagents were purchased from Sigma Chemical Co.

To avoid any misinterpretation of the data due to dimethyl sulfoxide (DMSO), used to dissolve atorvastatin, the appropriate volume of DMSO was added to the medium in control wells. We verified that the mean values of control cells (exposed to DMSO) were not significantly different from those of untreated cells (grown in medium alone; data not shown).

Cells

TUBO cells are a cloned line generated from a BALB-neuT mouse mammary gland carcinoma and express large amounts of neu protein [17]. N11 glial cells are a macrophage-like mouse cell line [18]. Cells were cultured in DMEM with 20% FBS in a humidified atmosphere at 37° C and 5% CO_2.

Mice and in vivo treatments

Six-week-old female BALB/c mice were from Charles River Italia SpA (Calco, Italy). Virgin female BALB-neuT mice transgenic for the transforming activated rat *Her2/neu* oncogene under the transcriptional control of the mouse mammary tumor virus promoter were bred under specific pathogen-free conditions by Charles River (Calco, Italy). Mice were treated according to the guide lines established in Principles of Laboratory Animal care (directive 86/609/EEC). All the experiments were approved by the institutional Ethical Committee.

Mice were randomLy assigned to control and treatment groups and all groups were treated concurrently. Atorvastatin was brought into suspension in phosphate buffered saline (PBS) at 12 μg/day, and a 0.2-mL volume (equivalent roughly to 40 mg/Kg/day in humans' treatment) was administered via oral gavage. Atorvastatin administration occurred once daily, 5 days per week for 8 weeks, starting from week 10 until week 17. Control mice were treated with PBS only.

BALB/c mice were challenged s.c. in the right flank with 0.2 mL of a suspension containing the minimal lethal dose of TUBO cells (10^5) [17]. BALB-neuT mice were inspected weekly to monitor the appearance of autochthonous mammary tumors. Neoplastic masses were measured with calipers in two perpendicular diameters and the average value was recorded. Progressively growing masses

>1 mm mean diameter were regarded as tumors. According to our ethical protocol mice were killed when a first tumor exceeded 10 mm mean diameter.

Mammary gland isolation.

BALB/c and BALB-neuT mice were sacrificed at week 18, when the atorvastatin treated groups were at the end of the 8 weeks atorvastatin-treatment period. The thoracic and abdominal mammary glands were isolated, incubated for 48 hours at 4°C in RNA Later (Quiagen, Hilden, Germany), drained and stored at -80°C for 1 day and than homogenized in MLB buffer (125 mM Tris-HCl, pH 7.4, 750 mM NaCl, 1% NP40, 10% glycerol, 50 mM $MgCl_2$, 5 mM EDTA, 25 mM NaF, 1 mM $NaVO_4$, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL aprotinin and 1 mM PMSF).

Mevalonate pathway activity.

Cells were incubated for 24 h with 1 µCi of [^{3}H]acetate (3,600 mCi/mmol; Amersham International, Piscataway, NJ), then washed with PBS and transferred in glass microcentrifuge tubes. The intracellular synthesis of cholesterol, FPP and ubiquinone was measured by methanol/hexane extraction method [19]. After the solvent evaporation, the lipid-enriched cellular extract was resuspended in 30 µl chloroform and resolved by thin layer chromatography (TLC) on silica gel (LK6D Whatmann; Merck, Darmstadt,

Germany), using a 1:1 diethyl ether/hexane solution as mobile phase. Intracellular isopenthenyl pirophosphate (IPP) was detected as previously reported [20], with the following modifications: the cellular suspension, rinsed with ice-cold acetonitrile containing 100 mM $NaVO_4$, was lyophilized under vacuum, re-suspended in 20 µl dimethylhexylammine and loaded on the TLC silica gel. The resolution was made in a 1:1 solution of 50% methanol/0.8 M ammonium formate (pH 7.4)/2 mM dimethylhexylammine. Standard solutions of cholesterol, ubiquinone, FPP and IPP were employed to identify each isoprenoid species. After the separation, the gel was exposed to iodine-saturated atmosphere. Each spot was cut and solubilised and the radioactivity incorporated was measured by liquid scintillation counting (Ultima Gold, Perkin Elmer). The results were expressed as fmol/1 x 10^6 cells, according to the titration curve previously obtained.

G-proteins activity

The GTP binding, which is considered an index of monomeric G-proteins activation [21], was measured as followed. For Ras-GTP binding cells were lysed in MLB buffer (125 mM Tris-HCl, 750 mM NaCl, 1% NP40, 10% glycerol, 50 mM $MgCl_2$, 5 mM EDTA, 25 mM NaF, 1 mM $NaVO_4$, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride PMSF; pH 7.5) and centrifuged at 13,000g for 10 min at 4°C. An aliquot of supernatant was

taken out for determination of proteins content and was directly probed with an anti-Ras antibody (1:250, in TBS-milk 5%, Millipore, Billerica, MA), to measure total Ras protein. 30 µg of the supernatant was incubated for 45 min at 4°C with the Ras Assay Reagent (Raf-1 RBD, agarose conjugate; Millipore). The beads were then washed 3 times in MLB buffer and harvested by the addition of 20 µl LaemmLi buffer (125 mM Tris, 4% w/v SDS, 20% v/v glycerol and 1% β-mercaptoethanol). Then samples were resolved by SDS-PAGE and Western blotting using the anti-Ras antibody to detect GTP-bound activated Ras. RhoA activation was detected using the G-LISA^{TM} RhoA Activation Assay Biochem Kit (Cytoskeleton Inc, Denver, CO): cells were washed with ice-cold PBS, lysed in 0.2 mL lysis buffer of the kit and centrifuged at 13,000 x g for 5 min. 10 µl of supernatants were taken off to measure the protein content, whereas the remaining part was used for the RhoA-GTP binding assays, according to the manufacturer's instructions. Absorbance was read at 450 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). For each set of experiments, a titration curve was prepared, using serial dilution of the Rho-GTP positive control of the kit. Data were expressed as mU absorbance/mg cell proteins. Rac activity was measured with the G-LISA^{TM} Rac 1/2/3 Activation Assay Biochem Kit (Cytoskeleton Inc), according to the manufacturer's instructions.

RhoA kinase activity

Rho kinase activity was measured using the CycLex Rho Kinase Assay Kit (CycLex Co., Nagano, Japan), a single site binding immunoassay, as previously reported [22]. For each set of experiments, a titration curve was set, using serial dilution of recombinant RhoA kinase (Rock2, MBL Inc, Woburn, MA) in kinase buffer. Data were expressed as mU absorbance/mg cell proteins.

Western blot analysis

TUBO cells and mammary glands homogenates were lysed in MLB buffer, sonicated on crushed ice (with two bursts of 10 s, Hielscher Ultrasonics GmbH, Teltow, Germany) and centrifuged at 13,000g for 10 min at 4°C. 20 µg cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore) and probed with the following antibodies: anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-extracellular signal-regulated kinase 1/2 (ERK1/2; diluted 1:1000 in TBS-milk 5%, Millipore); anti-ERK 1/2 (diluted 1:500 in TBS-milk 5%, Millipore); anti-phospho-Ser(473)-Akt (diluted 1:200 in TBS-milk 5%, Millipore); anti-Akt (diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology Inc, Santa Cruz, CA); anti-IkBα (diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology Inc); anti-GAPDH (diluted 1:500 in PBS-BSA 1%, Santa Cruz Biotechnology Inc). Membrane was washed in TBS-Tween 0.1%, subjected for 1 hour to a peroxidase-conjugated anti-rabbit or anti-mouse IgG (diluted

1:3,000 in TBS-Tween with blocker non-fat dry milk 5%, Bio-Rad), washed again with TBS-Tween 0.1%, and proteins were detected by enhanced chemiluminescence (Perkin Elmer, Waltham, MA).

NF-kB activity

Cells were rinsed with lysis buffer A (10 mM HEPES, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 mg/mL aprotinin, 2 mg/mL leupeptin, 0.1% NP-40, pH 7.6), incubated for 10 min on ice, vortexed and centrifuged for 30 s at 13,000 g to pellet nuclei, which were re-suspended in 0.2 mL wash buffer B (25 mM HEPES, 2 M KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 mg/mL aprotinin, 2 mg/mL leupeptin, pH 7.6) and incubated at 4°C for 10 min. An equal volume of buffer C (25 mM HEPES, 0.1 mM EDTA, 20% glycerol, pH 7.6) was added and after 10 min the mix was centrifuged at 13,000 g for 15 min at 4°C. The activity of NF-kB in the supernatants containing the nuclear extracts was assessed by the TransAM™ Flexi NFκB Family kit (Active Motif, Rixensart, Belgium), adding 1 pmol of the biotinylated NF-kB consensus site 5´-GGGACTTTCC-3´ to 10 µg of nuclear extracts. The absorbance at 450 nm was measured with a Packard EL340 microplate reader (Bio-Tek Instruments). For each set of experiments, a blank was prepared with bis-distilled water, and its absorbance was subtracted from that obtained in the presence of nuclear extracts. To assess the procedure specificity, a competition assay was

performed by adding 40 pmol of wild type non-biotinylated oligonucleotide to nuclear extracts derived from LPS-treated N11 cells and performing the assay as described above. Data were expressed as mU absorbance/mg cell proteins.

Cell proliferation and apoptosis assays

TUBO cells were seeded at density of $5x10^3$/mL into 96-well microplates flat bottom in 100/well in complete DMEM medium with 20% of FBS (5% CO_2, 37 °C, 95% humidity) and after 6 hours medium was replaced with serum-free medium. After over-night incubation, Br-deoxyuridine (10 μl/mL; Cell Proliferation ELISA, BrdU; Roche, Penzberg, Germany) was added in every well, while atorvastatin (100 μM) and parthenolide (10 μM), previously dissolved in DMSO as for manufacturer's instruction, or mytomicin-C (50 μg/mL) were added in the appropriate wells. After 12 h of incubation, cell proliferation was assessed using BrdU assay following the producer's instructions. The absorbance was evaluated by microplate reader (at wavelength of 370 nm). Growth of TUBO cells was represented as optical density (OD). For apotosis, TUBO cells treated in similar conditions were evaluated with the APC BrdU flow kit (BD Pharmingen, Bioscience, S. Diego, California) following manufacturer's instructions.

Statistical analysis

All data in text and Figures are provided as means ± SE. The results were analyzed by a one-way Analysis of Variance (ANOVA). Survival curves were analyzed by Chi square Test and the tumor growth was analyzed by Student's t Test. A $p < 0.05$ was considered significant.

RESULTS

Atorvastatin inhibits the mevalonate pathway activation and the activation of Ras and RhoA in TUBO cells

TUBO cells, a cell line established from BALB-neuT mice, were grown in the presence of atorvastatin, in order to analyze the metabolic effects of the statin in our experimental model: by inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A reductase enzyme, atorvastatin significantly reduced the *de novo* synthesis of isoprenoids such as IPP and FPP, and of cholesterol, the final metabolite of the mevalonate pathway. Also the rate of synthesis of ubiquinone, whose tail derives from the polymerization of isoprenoid units [23], was diminished in atorvastatin-treated cells (Figure 1A). As a consequence of the reduced isoprenoids synthesis, the GTP-binding of Ras (Figure 1B) and RhoA (Figure 1C), which is an index of the isoprenylation and activity of monomeric G-proteins [21], was lower in cells incubated with atorvastatin. This effect was consequent to the inhibition of the mevalonate cascade exerted by atorvastatin, since it was prevented by co-incubation with mevalonate (Figure 1B, 1C). We

then examined some downstream signaling pathways of Her2, focusing on MAP kinases ERK1 and ERK2, which are effectors of Ras, and on Akt, which can also be activated independently from Ras [13]. ERK1 and ERK2 were constitutive phosphorylated in TUBO cells and such activation was dramatically reduced by atorvastatin (Figure 1D). Again the addition of mevalonate, which *per se* had no effect on ERK1/2 phosphorylation, abolished the statin's effect. Also Akt was basally phosphorylated in TUBO cells, but the phosphorylation status was only slightly affected by atorvastatin. The statin did not change the total amount of Ras, ERK1/2 and Akt (Figure 2A, 2C). In parallel, atorvastatin reduced and mevalonate restored the activity of RhoA kinase (Figure 2B), which is one of the downstream effectors of the geranylgeranylated RhoA [21]. The farnesyl transferase inhibitor FTI-I and the geranylgeranyltransferase inhibitor GGTI-286 mimicked the effects of atorvastatin on the MAP kinases and on the RhoA/RhoA kinases pathways (Figure 2 A, 2B), respectively. Taken together these results suggest that the statin exert their typical metabolic effects – the reduction of the mevalonate pathway activity – in TUBO cells; by doing so, they lowered the synthesis of isoprenoids like FPP and GGPP and reduced the activity of Ras- and RhoA-dependent signaling pathways.

Atorvastatin abolishes the activation of Ras and MAP kinase in BALB-neuT mice mammary gland tumors

Female BALB/c mice and BALB-neuT mice were sacrificed at 18 weeks of age, a time at which in BALB-neuT mammary glands multiple tumors develop [24]. A sub-group of BALB-neuT mice was treated with atorvastatin 12 μg/day, a dose approximately comparable to that received by patients subjected to an anticholesterolemic therapy [16]. Mammary glands were collected and examined for the activity of the downstream effectors of Her2/neu. The expression of total Ras did not change between BALB/c and BALB-neuT mice (Figure 2A), whereas significant differences were detected in the active amount of Ras: indeed Ras-GTP was undetectable in non transformed mammary glands of BALB/c mice, but was present in tumoral glands of BALB-neuT mice. Interestingly, in BALB-neuT mice treated with atorvastatin, the activation of Ras was prevented (Figure 2A). Again the statin effect was not limited to Ras protein, but was evident on other isoprenylated monomeric G-proteins: for instance the activity of RhoA and RhoA kinase, which did not differ between BALB/c and BALB-neuT mice, were also reduced in statins-treated animals (Figure 2B). Also the geranylgeranylated Rac GTPase showed a similar behavior in the presence of statins (data not shown).

In keeping with the Ras-GTP activity, phosphorylated active ERK1/2 were hardly detected in BALB/c mammary glands, but were constitutively present in tumor glands of BALB-neuT mice; again atorvastatin strongly reduced the phosphorylation of ERK1 and ERK2 in BALB-neuT tumor extracts (Figure 2C).

Interestingly Akt was basally phosphorylated in BALB/c mice; such a phosphorylation was higher in BALB-neuT mice and was slightly reduced by the statin (Figure 2C). The total amount of ERK1/2 and Akt did not change between wild type and transgenic mice under any experimental conditions (Figure 2C). Therefore atorvastatin slowed down the MAP kinases pathway, which was constitutively activated in the tumor tissue, and reduced the phosphorylation of Akt; these data lead us to hypothesize that the HMGCoAR inhibitor might have an antitumor effect on BALB-neuT mice.

Atorvastatin administration does not inhibit the growth of implanted TUBO cells and the spontaneous tumor onset in BALB-neu T mice

Implanted TUBO cells grow fast in wild-type BALB/c mice and form a palpable tumor mass in about 2 weeks [25]. To evaluate the effects of atorvastatin on the growth of TUBO tumors, female BALB/c mice were challenged s.c. with 10^5 TUBO cells. On the same day, the oral treatment with atorvastatin was started. No significant differences were observed in the growth of TUBO tumors in treated versus control mice (Figure 3A, B). Only in 2/6 atorvastatin treated mice, a delay in tumor growth was observed (Figure 3C).

Female BALB-neuT mice develop mammary tumors in all their mammary glands. Mammary cells overexpressing Her2/neu give rise to atypical hyperplasia around week 3 of age. At week 7, the hyperplasia progresses to multifocal

preneoplastic lesions [24]; in our control group at least one palpable carcinoma (>1 mm mean diameter) was evident in each control mice by week 24 (Figure 3D), while by week 35 a tumor mass was palpable in all 10 mammary glands or the tumor burden was such that the mice were sacrificed for ethical reasons (Figure 3E). Oral administration of atorvastatin from week 10 to week 17 did not delay tumor progression in female BALB-neuT mice. All treated mice had at least one palpable tumor by week 25 (Figure 3D) and at 37 weeks all animals were dead (Figure 3E).

These findings show that oral daily administration of atorvastatin does not inhibit the in vivo growth of both transplantable and autochthonous Her2/neu tumors.

By inhibiting the mevalonate pathway, atorvastatin induces the activation of NF-kB in TUBO cells

Strict correlations exist between the rate of mevalonate pathway and the activation of the transcription factor NF-kB [26,27] and were present also in TUBO cells: indeed atorvastatin induced a significant increase of the nuclear translocation of NF-kB (Figure 4A), which was accompanied by the reduction of the inhibitory protein IkBα (Figureure 4B). These modifications were similar to those induced by the bacterial lipolysaccharide from *E. coli* in microglial N11 cells, chosen as a model of cells highly responsive to the inducers of NF-kB

(Figure 4A, 4B). Again, the effects of atorvaststin were prevented by the co-incubation with mevalonate, suggesting that the activation of NF-kB was negatively controlled by the metabolites of the mevalonate cascade in TUBO cells. In particular, geranylgeraniol (10 µM for 24 h) and, at a lesser extent, farnesol (10 µM for 24 h), two cell permeant analogues of the GGPP and FPP, abrogated the atorvastatin-induced translocation of NF-kB (data not shown).

NF-kB is a crucial factor for cell proliferation and survival [28] and we wondered whether its activation could sustain cell proliferation also in TUBO cells. Indeed atorvastatin-treated cells, despite the reduction of the proliferative/pro-survival signals exerted by ERK1/2 and Akt (Figure 1D), did not show a lower proliferation respect untreated cells (Figure 4D). However, if we prevented the activation of NF-kB with its specific inhibitor parthenolide (Figure 4C), statin was able to reduce TUBO cells proliferation (Figure 4D) and increase the apoptotic rate (data not shown). This result suggests that the inhibition of MAP kinases and Akt kinase pathways was not sufficient to down-regulate the proliferation of TUBO cells, if we did not suppress also the NF-kB signaling.

DISCUSSION

The pleiotropism of statins in mammalian cells make them versatile drugs, currently used or under clinical trials in several cardiovascular, autoimmune and neurodegenerative diseases [1]. Cancer represents a putative field of

application for statins: besides the high need of cholesterol which a fast proliferating cell requires for the synthesis of its membranes, most activities of transformed cells, such as proliferation, matrix invasion and angiogenesis, are controlled by small G-proteins belonging to the Ras and Rho family, which must be isoprenylated to activate their downstream effectors [2]. By reducing the synthesis of cholesterol and isoprenoids, statins may theoretically hamper all these functions in tumor cells. As far as breast cancer is concerned, statins have been reported to impair the tumor growth and invasion, and enhance the efficacy of anticancer drugs [7,8]. On the other hand, it is not clear whether statins could also reduce the incidence of breast tumors: until now the few epidemiologic studies have produced conflicting results [10,12], although recent works have shown that the HMGCoAR inhibitors prevent the onset of estrogen-receptor negative tumors [11] and reduce the proliferation of the most aggressive isotypes of breast cancers [29].

The subgroup of Her2 overexpressing breast cancers is a particularly interesting model to evaluate the statins effects, because one of the most active downstream transducer of the Her2 oncogene is Ras GTPase, whose activity is strictly dependent on the mevalonate pathway [7]. Recently the combination of fluvastatin with the Her2 targeting monoclonal antibody trastuzumab has been successfully employed to enhance the apoptotic rate in the Her2 overexpressing SK-BR-3 cells [8]. Another study reported a direct antiproliferative effect of

lipophilic statins in a mouse Her2 positive cell line implanted in FVB-NeuN mice [4].

In our work we investigated the effect of atorvastatin on BALB-neuT mice, in which invasive mammary tumors occur with a high penetrance. Such experimental model gave us the opportunity to monitor the tumor progression directly in mammary glands, thus analyzing the effect of statin on a tumor developing in its "physiological" site and in an immunocompetent host. We treated animals with atorvastatin 12 μg/day, corresponding to a standard protocol of approximately 40 mg/Kg/day atorvastatin for patients treated for hypercholesterolemia. Such a dose results in a serum concentration in the high micromolar range, which has been associated to an appreciable antitumor activity [4].

In the epithelial TUBO cell line, derived from the Her2 overexpressing transformed cells of BALB-neuT mammary tumors, atorvastatin effectively inhibited its natural target HMGCoAR, thus decreasing the rate of synthesis of several metabolites produced in the mevalonate pathway, like the isoprenoids FPP and IPP and the end-product cholesterol. Also the side chain of ubiquinone, which acts as an electron shuttle between NADH dehydrogenase (Complex I; EC 1.6.5.3) and ubiquinol-cytochrome c reductase (Complex III; EC 1.10.2.2) in the mitochondrial respiratory chain, contains an isoprenoid structure[23]. Also the amount of ubiquinone was significantly lowered by atorvastatin in our model.

An adequate content of ubiquinone is mandatory for the efficient activity of the mitochondrial chain, which ultimately leads to the synthesis of ATP and we may suppose that statin-treated TUBO cells have a lower supply of ATP. Interestingly in MDA-MB-231 breast cancer cells lovastatin has lowered the activity of glicolysis and citric acid cycle, two metabolic pathways involved in ATP production, and this event has been related to a decreased cell proliferation [6].

By lowering FPP levels, atorvastatin greatly reduced the activity of Ras, a downstream effector of Her2 oncogene which was constitutive active in TUBO cells. Untransformed mammary glands of BALB/c mice were devoid of active GTP-bound Ras, which was instead detected in glands of BALB-neuT animals collected at 18 weeks, when palpable tumors arose in most glands. Interestingly atorvastatin abrogated the Ras-GTP binding in the tumor mammary tissue of BALB-neuT mice. In parallel, by limiting the cell amount of isoprenoids, the drug reduced the activity of the geranylgeranylated RhoA protein, a second small GTPase present in TUBO cells and in tumor glands, and of its downstream effector RhoA kinase. Although RhoA is often overexpressed in breast cancers and is crucial for breast epithelium proliferation and invasion [6], its activity did not change between BALB/c and BALB-neuT mice, suggesting that RhoA activation does not play a critical role in the transformation of cells driven by Her2 oncogene. The striking difference in Ras activity between wild-type and

transgenic mice lead instead to hypothesize that Ras has a prominent role in the tumorigenesis and/or in the tumor growth in BALB-neuT mice.

In cells with constitutively activated EGF receptors, the MAP kinases pathway induce an increased proliferation, allowing tumor cells to increase cyclin D1 expression and to progress into cell cycle [4,30]. The activation of ERK1 and ERK2 kinases in our experimental model strictly follows the activity of Ras: phospho-ERK1/2 were absent in BALB/c non transformed cells, present in BALB-neuT tumor glands and in TUBO cells, and was markedly reduced by atorvastatin. On the other hand, the activity of the PI3K/Akt pathway, another downstream effector of Her2 receptor, displayed minor changes in the presence of the statin: a small amount of phosphorylated active Akt was detectable also in non transformed mammary glands, which are devoid of constitutive active Her2. Moreover phospho-Akt was slightly increased in BALB-neuT animals and slightly reduced by atorvastatin. These results are not surprising: for example in MCF-7 cell line, where Her2 is absent, Akt is constitutive phosphorylated [31], probably in consequence of the multiple activating signals converging on kinase B, beside those linked to Her2 and EGF receptors. Akt can also be a Ras-independent effector of Her2 oncogene [13], thus providing an explanation of the limited effects of atorvaststin on Akt activity. Indeed an effective reduction of Akt activity has been achieved by lovastatin only if associated to the EGF receptor-targeting drug gefitinib [30]. It is actually supposed that PI3K/Akt

pathway plays a prevailing anti-apoptotic role in Her2 positive cells, whereas MAP kinases pathway has more pronounced effects on cells proliferation and tumorigenesis [30]. Since atorvastatin exerted a striking inhibition of Ras and ERK1/2 activity and produced also a little reduction of Akt activation in the mammary glands of BALB-neuT mice, it should have at least two requisites to effectively reduce the tumor growth in BALB-neuT animals.

Unexpectedly, we did not observed any growth reduction of tumor cells implanted in statin-treated BALB/c mice. Similarly atorvastatin did not delay the growth of the autochthonous mammary tumors in BALB-neuT animals, showing that it has not any chemopreventive effect on the onset of Her2/neu positive breast cancers.

Aggressive tumors often have redundant signaling pathways to increase proliferation and/or to escape apoptosis, thus having an enormous proliferative advantage towards non-transformed cells. A critical factor for cell proliferation and inhibition of apoptosis in mice, as well as in human cells, is the transcription factor NF-kB. In non-stimulated cells NF-kB is retained in cytosol by the inhibitor IkBα protein; on the contrary, cytokines, bacterial lipopolysaccharide, oxidative stress or drugs induce the phosphorylation and the successive ubiquitinylation of IkBα, allowing NF-kB to translocate into the nucleus [28]. The relationship between statins and NF-kB pathway is still a matter of debate and is highly variable depending on cell type. For example simvastatin has reduced the

phosphorylation and the degradation of IkBα, preventing the NF-kB-mediated osteoclastogenesis [32]; the same drug has decreased the activation of NF-kB, de-repressed the transcription of PTEN gene, reduced the phosphorylation of Akt and the proliferation of breast cancer cells MDA-MB-231 cells [5]. On the opposite hand, statins have enhanced the translocation of NF-kB in glial cells [33] and in adipocytes [34], two models where the NF-kB pathway was inhibited by RhoA protein. Interestingly it has been reported that the activity of NF-kB can be reduced by isoprenoids derived from mevalonate pathway, such as FPP [27] and GGPP [26].

In our experimental conditions, atorvastatin increased the nuclear translocation of NF-kB, by reducing the amount of the inhibitory protein IkBα. The statin effect was due to the inhibition of HMGCoAR, beacuse it was abolished by mevalonate and by intermediate metabolites, like FPP and GGPP analogues. This observation suggests that the statins effect on NF-kB are likely due to the decrease of G-proteins activity.

When active, NF-kB up-regulates several genes involved in the inhibition of apoptosis and/or in cell proliferation, such as Bcl2, Bcl-xl, cyclin D1, cyclin D2, c-Myc, c-Myb; moreover it increases the synthesis of cytokines and growth factors, which sustain the tumor proliferation and progression with autocrine mechanisms [28]. Therefore a tumor cell with high activity of NF-kB is able to survive and proliferate, although other proliferation signals are turned down.

This behavior may explain why atorvastatin, which decreased the activity of the downstream effectors of Her2 oncogene like Ras, ERKs and Akt proteins, and would have appeared a promising drug to inhibit cell proliferation, resulted devoid of efficacy in reducing tumor development in BALB-neuT mice and TUBO cells proliferation. Interestingly, only when we prevented the activation of NF-kB with parthenolide, the statin became able to lower cells proliferation and increase apoptosis. Our results show that the signaling pathways dependent on Her2/neu oncogene, like MAP kinases and Akt kinase pathways, do not fully explicate the growth of Her2/neu positive tumors. On the other hand, neither NF-kB alone seems sufficient to sustain the proliferation of Her2/neu overexpressing cells: indeed the inhibition of NF-kB with parthenolide did not reduce significantly TUBO cells proliferation. Only the simultaneous suppression of Her2/neu-dependent pathways, as well as of NF-kB, achieves a good antiproliferative effect. The redundancy of pro-proliferative signals may explain the aggressive phenotype of Her2/neu positive tumors, a feature frequently observed in clinical practice.

CONCLUSIONS.

Our results show that statins can contemporarily modulate multiple signaling pathways involved in cell death and proliferation in Her2/neu positive mammary tumor cells. Such pleiotropism, which in other contexts has made statins drugs potentially useful in several diseases, may paradoxically dissipate the expected beneficial effects of HMGCoAR inhibitors in terms of cell proliferation. The identification of each pathway controlling proliferation is mandatory to take advantage from the use of statins in association with tumor-specific and selective targeted therapies. The use of statins in cancer has often produced conflicting results, depending on the tumor types investigated and on the experimental models chosen. Differently from other *in vivo* models, in which statins have effectively reduced the growth of xenografted or subcutaneously implanted Her2/neu positive mammary cancer cells [4], our work has monitored the natural development of Her2/neu positive tumors in the mice mammary gland, thereby allowing the tumor to interact with its usual microenvironment and to be physiologically supported by local stimuli derived from growth factors, cytokines and stromal cells. All these factors may be determinant to condition the response of transformed cells to anticancer drugs. At the light of our results, we believe that many other extensive investigations, considering the complex interaction between tumor and its microenvironment should be performed before raising any conclusion on the potential anticancer efficacy of statins.

LIST OF ABBREVIATIONS: HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IPP, isopenthenyl pyrophosphate; MAPK, mitogen-activated kinase; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; PS, penicillin-streptomycin; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; BrdU, Br-deoxyuridine; NF-kB, nuclear factor kB.

COMPETITING INTERESTS.

The authors declare that they have no competiting interests.

AUTHORS' CONTRIBUTION.

CR and HP carried out the in vitro experiments and the animals treatment, performed the statistical analysis and wrote the manuscript; EB carried out the in vivo experiments; CDB performed the biochemical assays; RAC participated to the design and coordination of the study; AB and FC conceived and coordinated the study and revised the manuscript.

All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Effect of atorvastatin on mevalonate pathway, Ras, RhoA, ERK1/2 and Akt in TUBO cells.

Cells were incubated in the absence (*CTRL*) or presence of atorvastatin (100 μM for 24 h, *AT*), then subjected to the following investigations.

A. Measurement of the mevalonate pathway activity. Cells were grown in a medium containing 1 μCi of [^{3}H]-acetate, then lysed and subjected to lipidic extraction. IPP, FPP, cholesterol and ubiquinone were detected as described under Materials and Methods. Data are presented as means $\pm$ SE (n = 3). Vs CTRL: *$p < 0.05$.

B. Ras-GTP pull down. Cells were lysed and the expression of Ras-GTP and total Ras was analyzed as described in the Materials and Methods section. The farnesyl transferase inhibitor FTI-I (10 μM for 24 h, *FTI*) was added as a positive control of Ras inhibition. The Figure is representative of 3 experiments with similar results.

C. RhoA-GTP and RhoA kinase activity. Samples were subjected to ELISA assay to measure the amount of RhoA-GTP (*open bars*) and the activity of RhoA kinase (*hatched bars*). The geranylgeranyltransferase transferase inhibitor GGTI-286 (10 μM for 24 h, *GGTI*) was added as a positive control of RhoA inhibition. When indicated, 2 ng of recombinant active RhoA-GTP protein (*rRho*) was added to the cells lysates derived from atorvastatin-treated cells; this competition assay was

taken as an index of the specificity of the assays. The experiments were performed in duplicate, as described under Materials and Methods. Data are presented as means ± SE (n = 3). Vs CTRL: ***** $p < 0.005$; vs AT: **°** $p < 0.05$.

D. Western blot detection of phospho-ERK1/2, ERK1/2, phopsho-Akt, Akt and GAPDH. Mammary glands homogenates were analyzed by Western blotting with an anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-ERK1/2, an anti-ERK1/2, an anti-phospho-Ser(473)-Akt or an anti-Akt antibody, as reported in the Materials and Methods section. The expression of GAPDH was used to check the equal protein loading. The Figure is representative of 3 experiments with similar results.

Figure 2. Effect of atorvastatin on Ras, RhoA, MAPK and Akt in mammary glands of BALB/c and BALB-neuT mice.

BALB/c and transgenic BALB-neuT mice were treated with 40 mg/kg/day of atorvastatin for 8 weeks; then isolated mammary glands for protein extraction. As control were used both transgenic BALB-neuT mice and wilde type BALB/c mice.

A. Ras-GTP pull down. Mammary glands homogenates were lysed and the expression of Ras-GTP and total Ras was analyzed as described under Materials and Methods. The Figure is representative of 3 experiments (for a total of 5 mice in each group) with similar results.

B. RhoA-GTP and RhoA kinase activity. Samples were subjected to ELISA assay to measure the amount of RhoA-GTP (*open bars*) and the activity of RhoA kinase (*hatched bars*). The experiments were performed in duplicate, as described under Materials and Methods. Data are presented as means $\pm$ SE (n = 3). Vs CTRL neuT: ***** $p < 0.005$.

C. Western blot detection of phospho-ERK1/2, ERK1/2, phopsho-Akt, Akt. Mammary glands homogenates were analyzed by Western blotting with an anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-ERK1/2, an anti-ERK1/2, an anti-phospho-Ser(473)-Akt or an anti-Akt antibody, as described under Materials and Methods. The expression of GAPDH was used to check the equal protein loading. The Figure is representative of 3 experiments (for a total of 5 mice in each group) with similar results.

Figure 3. Effect of atorvastatin on the growth of Her2/neu over-expressing tumors.

A. Tumor latency (time required from the challenge to a tumor of the indicated mean diameter) in mice challenged with Her2/neu over-expressing TUBO cells and daily treated whit atorvastatin. BALB/c mice were challenged with a lethal dose (10^5 cells) of syngenic TUBO mammary tumor cells and orally treated with 40 mg/kg/day of atorvastatin administered by gavage 5 days/week (atorvastatin

n=6) for 8 weeks. Tumor diameters in treated mice were not significantly different in the atorvastatin treated mice compared with PBS treated mice (controls n=4).

B. Tumor growth (mean ± SE, mm) in function of time (days) in control and atorvastatin treated BALB/c mice, as in A.

C. Graph representing tumor growth in single BALB/c mice challenged with TUBO cells.

D. Percentage of tumor free control (n=14) and atorvastatin treated (n=8) BALB-neuT mice. Mice were cronically treated with 40 mg/kg/day of atorvastatin. Chi square test: 2.160 (not significant).

E. Overall survival of BALB-neuT mice as in C. Chi square test: 1.609 (not significant).

Figure 4.

Effects of atorvastatin and NF-kB on cells proliferation.

Cells were incubated in the absence (*CTRL*) or presence of atorvastatin (100 µM for 24 h, *AT*), then subjected to the following investigations. When indicated, the inhibitor of NF-kB parthenolide (10 µM for 24 h, *PART*) was added. Murine

microglial N11 cells, incubated with LPS (2 μg/mL for 24 h, *LPS*), were chosen as a positive control of NF-kB activation.

A. NF-kB activation by atorvastatin. The activity of NF-kB was detected in the nuclear extracts using an ELISA assay. In the competitions assay, used as a control of the specificity of the assay, the activity of NF-kB in LPS-treated cells was 2.55 ± 0.45 mU/mg prot. Measurements were performed in duplicate and data are presented as means ± SE (n = 3). Vs CTRL: ***** $p < 0.01$; vs AT: **°** $p < 0.02$.

B. Western blot detection of IkBα protein in cytosolic extracts. TUBO cells were lysed and analyzed by Western blotting with an anti-IkBα, as reported in the Materials and Methods section. The expression of GAPDH was used to check the equal protein loading. The Figure is representative of 3 experiments with similar results.

C. NF-kB activity in the presence of parthenolide. The activity of NF-kB was detected in the nuclear extracts as reported above. Measurements were performed in duplicate and data are presented as means ± SE (n = 3). Vs CTRL: ***** $p < 0.01$; vs AT: **°** $p < 0.001$.

D. TUBO cell proliferation. Proliferation of TUBO cells was detected measuring the BrdU incorporation at different time points. When indicated, the cell proliferation blocker mytomicin C (50 μg/mL **)** was used. Measurements were performed in quadruplicate and data are presented as means ± SE (n = 3). Vs CTRL: ***** $p < 0.01$; vs AT: **°** $p < 0.001$.

Figure 1

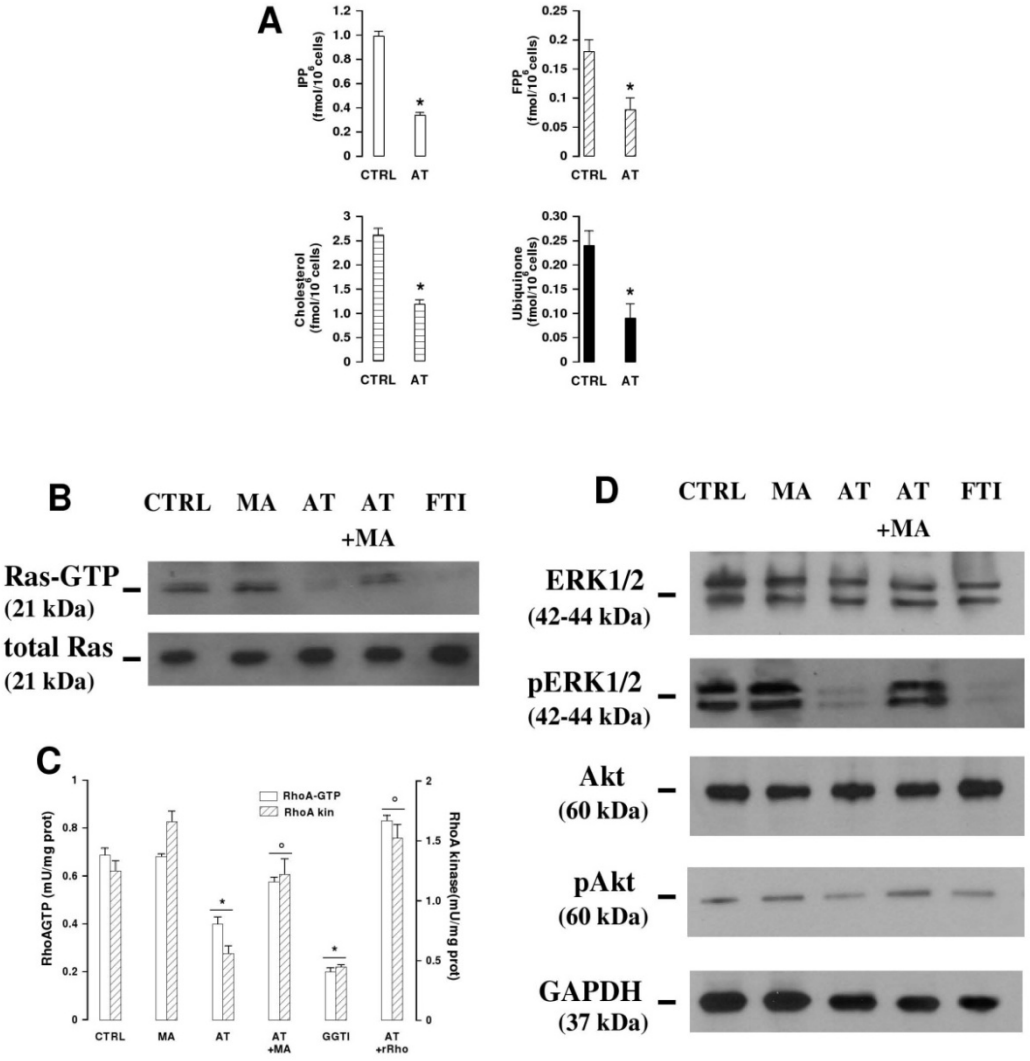


Figure 1

Figure 2

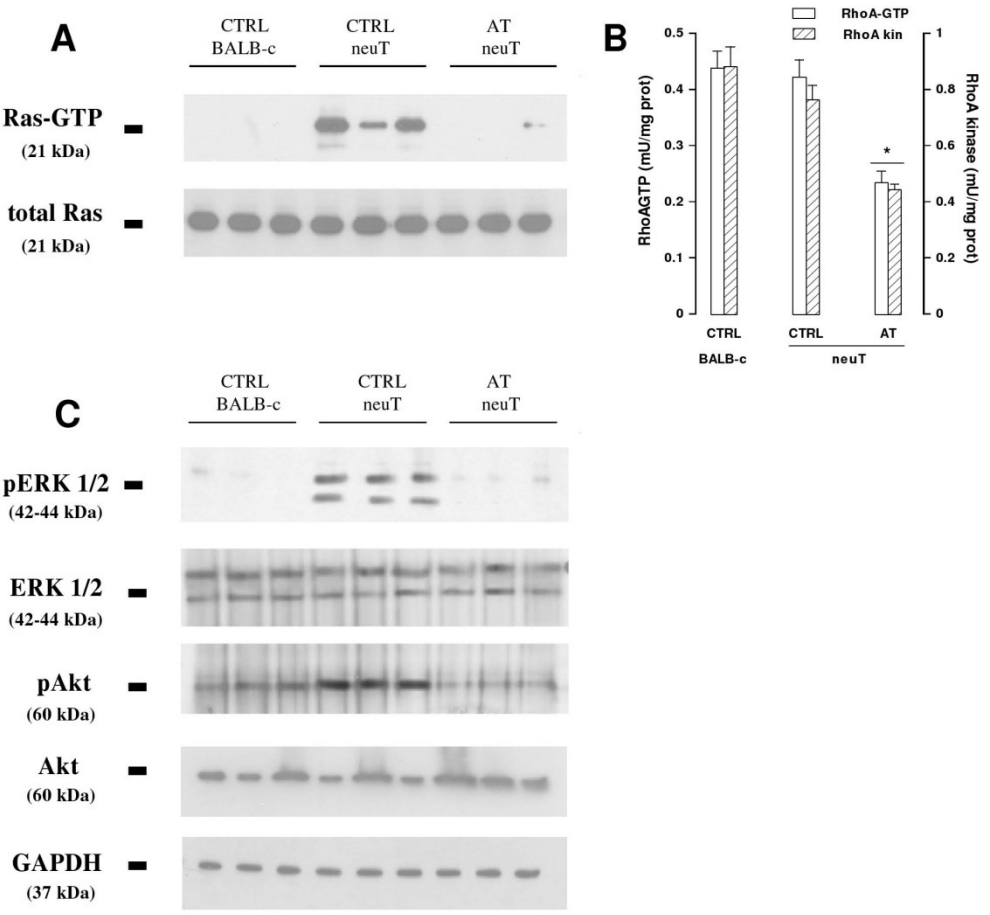


Figure 2

Figure 3

A

Latency (mm)	**Atorvastatin treated mice (days after tumor challenge)**	**Untreated mice (days after tumor challenge)**	
2.5	19±4	18±4	p= 0.158 n.s.
5	31±8	25±6	p= 0.263 n.s.
7	43±19	28±5	p= 0.260 n.s.

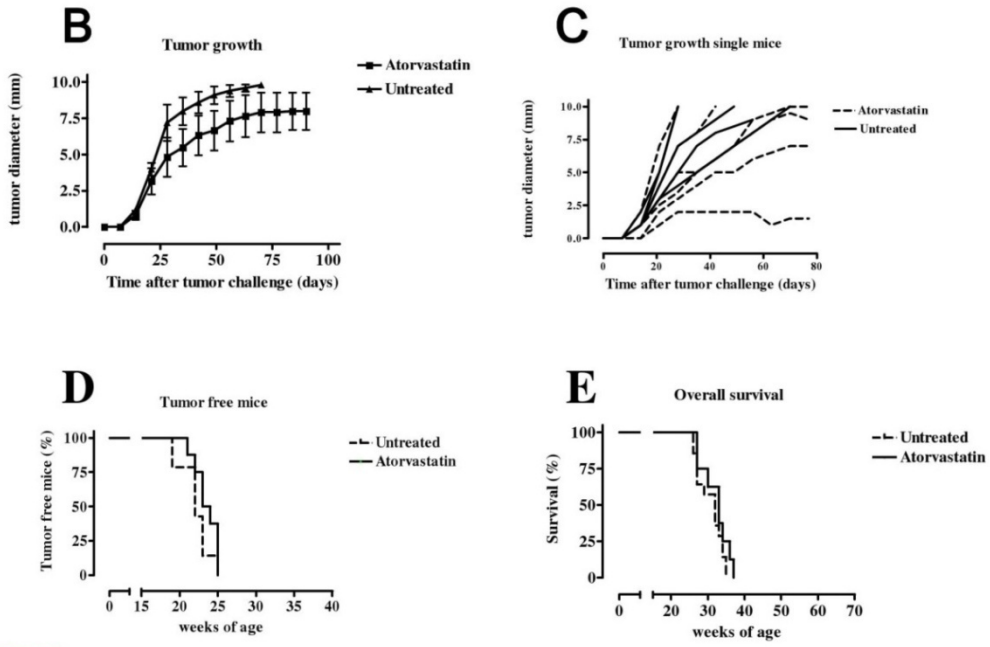


Figure 3

Figure 4

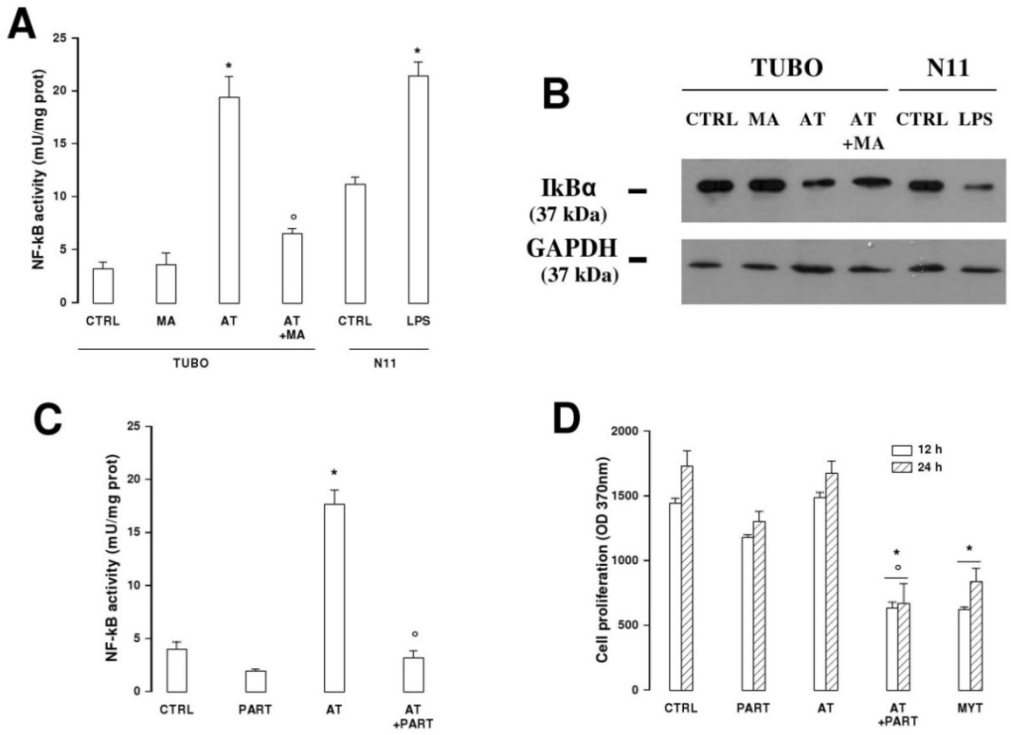
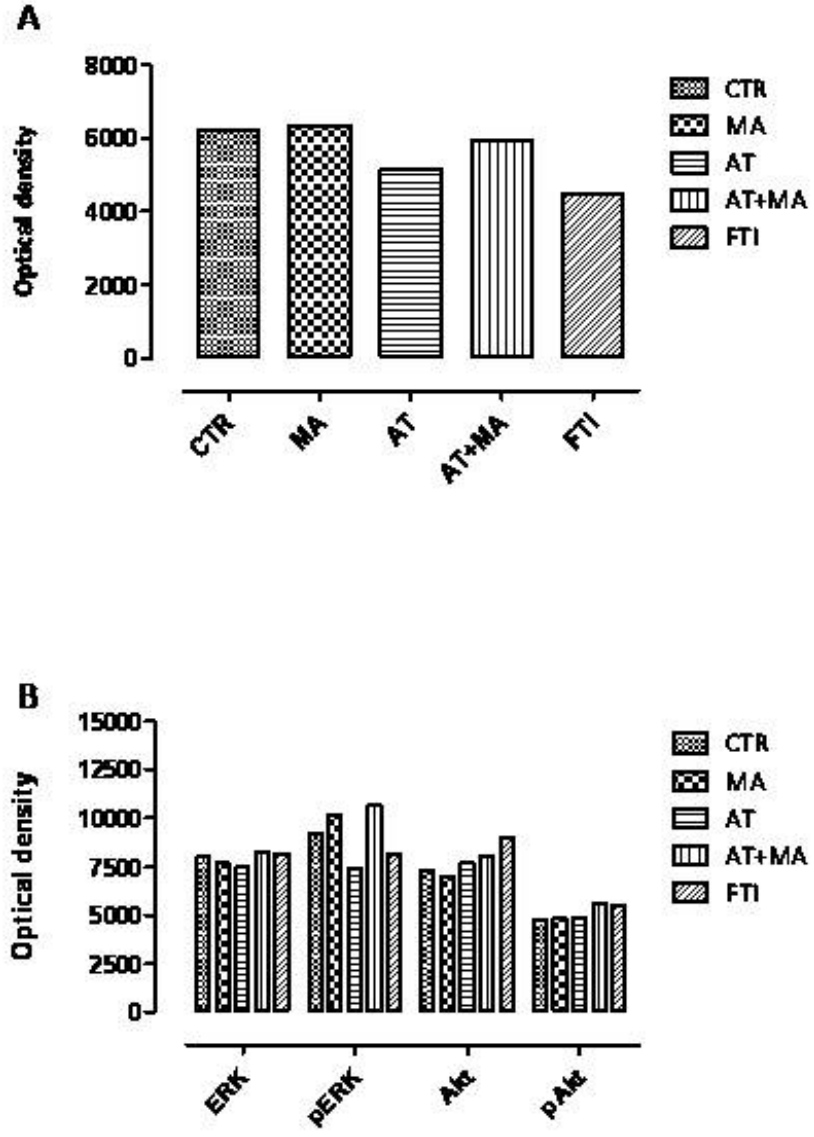
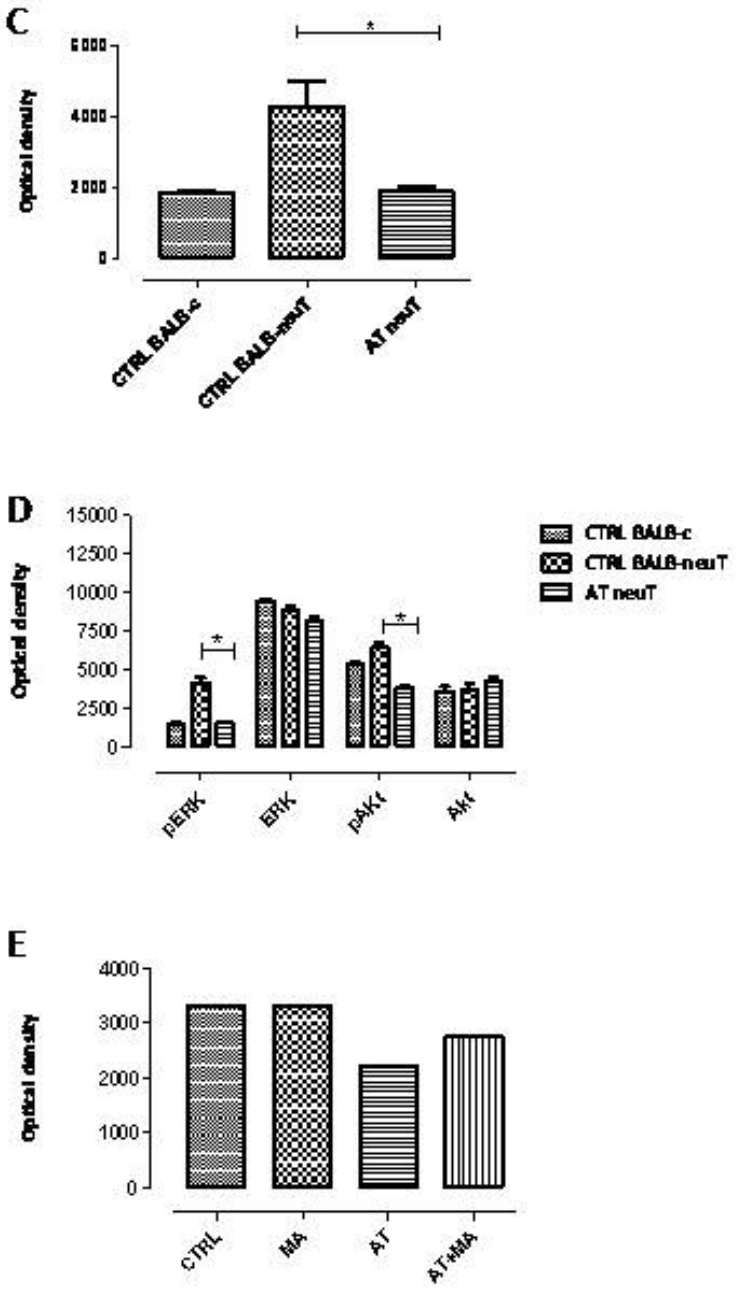


Figure 4

Supplemental figure 1




C
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pAKT
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CTRL BALB-neuT
AT neuT
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CTRL
MA
AT
AT+MA

Supplemental figure 1

Quantification of western blotting signals was obtained by densitometric analysis of the signal product Optical density . Proteins expression is normalized to the GAPDH signal from the same western blotting product. (A) Ras-GTP pull down in TUBO cells. (B) Western blot detection of ERK1/2, phospho-ERK1/2, Akt, phopsho-Akt. (C) Ras-GTP pull down on mammary glands; data are presented as means + SE (n = 3) vs CTRL * $p<0.0299$. (D) Western blot detection of phospho-ERK1/2, ERK1/2, phopsho-Akt, Akt on mammary glands; data are presented as means + SE (n = 3) pERK vs AT neuT * $p<0.0183$, pAkt vs AT neuT *$p<0.0272$. (E) Western blot detection of IkBα protein in cytosolic extracts of TUBO cells.

In the first part of this study we have describe the effects exerted by atorvastatin administration on TUBO cells. The data obtained show that this drug not inhibit proliferation on mammary tumor cells when administred alone. In the light of these results we have decided to investigate the functionality of this statin on cancer stem cells.
Furthermore, the analysis of gene expression of cancer stem cells derived from TUBO cells showed the existence of genes linked to statin by cholesterol syntesis.

2) In the second part, we detected transcripts associated with mouse and human mammary CIC. Among these genes we observed the presence of CIC genes associated to pathways affected by atorvastatin. We also detected xCT as a gene specifically upmodulated in CIC and we evaluated the effect of its inhibition on CIC characteristics, e.g. mammosphere formation and metastasis onset in animal models. The results obtained are in the paper titled "**Mouse-human microarray data integration reveals xCT as an oncoantigen of breast cancer initiating cells"** submitted for publication and under review in the journal " Cancer Research

Here we reported that an epithelial cell line derived from a BALB-neuT carcinoma (TUBO cells) generates mammospheres that can be propagated for serial *in vitro* passages. These mammospheres express markers associated with CIC phenotype, and efficiently generate tumors when injected in syngeneic mice. Mammospheres generated from TUBO cells displayed clonogenicity, self renewal, CIC markers and ability to differentiate in mammary epithelial cells and maintained the tumorigenic potential. To identify transcripts associated with mouse and human mammary CIC, we detected the genes differentially expressed between TUBO cells and mammospheres, identifying a total of 452 differentially expressed transcripts . We focused our attention on a subset of 183 transcripts which expression increased during mammosphere culture.

To define the oncoantigenic potential (an oncoantigen is a tumor-associated molecules that drive the progression of a neoplastic lesion from one stage to the next [Lollini et al. 2006]), of this set of genes we have devised a ranking procedure based on the availability of microarray cancer expression/clinical data and normal tissues expression data.

The ranking procedure is based on two steps: ranking on the basis of clinical outcome (CO score) and ranking on the basis of expression in normal tissues (NOR score).

To calculate CO score seven breast cancer public available microarray studies (GSE2034, GSE1456, GSE2990, GSE3494, GSE6532, GSE7390, GSE11121, 1500 arrays) encompassing distant metastasis outcome were used. To calculate the NOR score the Body Index data set (GSE7307, 512 arrays representing 82 adult tissues) was used.

From this analysis we identified two new breast CIC OAs: TMPRSS4 and xCT. Both genes are linked to invasion, migration and metastasis. For xCT we have proved its causal role in CIC maintenance. We have also identify a set of genes associated to Atorvastatin by Ingenuity knowledge based analisys, and we

observed that statin is activey interfering with the formation of mammospheres. We are currently evaluating the in vivo effect of statin on metastasis onset mediated by CIC in animal model.

Mouse- human microarray data integration reveals xCT as an oncoantigen of breast cancer initiating cells.

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Authorship note: *equally contributed, [§] equally supervised this work.

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Running title: xCT is an oncoantigen of breast cancer initiating cells

Key words: Oncoantigens, breast cancer, cancer initiating cells, microarray analysis, xCT

Abstract

Cancer initiating cells (CIC) constitute a self-renewing stem cell-like population responsible for the progression, metastasis, resistance to treatment, and recurrence of several tumors. An epithelial cell line (TUBO cells) derived from a mammary carcinoma arising spontaneously in BALB/c mice transgenic for the activated rat ERBB2 oncogene generates mammospheres expressing markers associated with CIC phenotype, and forms tumors in syngeneic mice. Comparison of the transcription profile of TUBO cells with that of their derived mammospheres, and meta-analysis of seven human breast tumor and one normal tissue microarray data sets led to the identification of a gene signature associated to mammary CIC, whose examination resulted in fresh indications on new potential oncoantigens and interaction of CIC with pharmaceutical compounds.

Among the identified oncoantigens, xCT, a 12-pass transmembrane amino acid transporter, plays a causal role in CICs maintenance. The role of xCT as CIC oncoantigen is endorsed by the ability of its functional inhibitor sulfasalazine

(SASP) to impair the formation of mammospheres *in vitro* and to inhibit lung metastasis formed by mammosphere cells injected intravenously.

These finding could be the basis for the design of new anti-cancer treatments aimed at CIC eradication.

Introduction

Recent data show that anti-cancer vaccines are uniquely effective in preventing the progression of early stages of cancer or the recurrence of residual disease (1). When the reaction elicited is directed against a protein required for the neoplastic progression (an oncoantigen), its evasion by a tumor is less likely (2). It is thus conceivable that the identification of new oncoantigens directly involved in the maintenance of the stem-cell like features of CIC could open a promising therapeutic opportunity for anti-cancer vaccines.

Many human malignancies are thought to be organized in a hierarchical network consisting of a few slowly dividing CIC, rapidly dividing amplifying cells, and differentiated tumor cells (3). The stem cell-like properties of CIC (self-renew and re-establishment of tumor heterogeneity) place them at the summit of this hierarchy (4) and make them responsible for tumor progression, metastasis, resistance to treatment, and recurrence (5, 6). Their existence has been shown in malignant tumors of many kinds, including breast carcinoma (7-10).

The notion that cancer progression may be driven by CIC has important implications. It often explains why current treatments fail to consistently eradicate malignant tumors. Those designed to shrink the bulk of a tumor, for example, may fail to eliminate the small fraction of CIC endowed with chemo- and radio-resistance and immunoevasive features (6). Instead, the final success

of a treatment and of a vaccine designed to prevent recurrence may rest on complete or functional CIC eradication.

Mammary stem cells down-regulate cell-cell junctions, display mesenchymal behavior (11, 12) *in vitro*, and survive and proliferate in anchorage-independent conditions in the form of floating spherical colonies termed mammospheres (13). CIC from human and mouse mammary tumor specimens, metastases, and cell lines can be isolated in function of their ability to grow as non-adherent spheres (14-18).

We have shown that mammary carcinomas spontaneously arising in mice transgenic for the activated rat ERBB2 oncogene (BALB-neuT mice) contain a population of sphere-generating cells endowed with the ability to initiate tumor *in vivo* (19). Here we report that an epithelial cell line derived from a BALB-neuT carcinoma (TUBO cells) (20) generates mammospheres that can be propagated for serial *in vitro* passages. These mammospheres express markers associated with CIC phenotype, and efficiently generate tumors when injected in syngeneic mice.

To identify transcripts associated with mouse and human mammary CIC, we integrated mouse-human data emerging from genome-wide transcription profiling, a strategy recently described for the selection of oncoantigens expressed during mammary carcinogenesis (21). Comparison of the transcription profile of TUBO cells cultured as epithelial monolayer (E cells) with

that of first three *in vitro* passages of their derived mammospheres, and the meta-analysis of seven human breast tumor and one normal tissue microarray data sets led to the identification of a gene signature associated with mammary CIC. Examination of this signature led to the isolation of a set of putative CIC oncoantigens whose assessment via meta-analysis of normal and cancer human transcription profiling data identified a list of mammary oncoantigens associated with CIC. Among them xCT, a 12-pass transmembrane heteromeric amino acid transporter (22) linked to invasion, migration and metastasis (23-27), plays a causal role in CIC maintenance as demonstrated in the present paper both *in vitro* and *in vivo*.

Materials and Methods

Cell and mammosphere culture

E cells, i.e. TUBO cells grown in adherent conditions as epithelial monolayer, were generated from a mammary carcinoma arising in a BALB-neuT female mouse (28), and cultured in epithelial medium, Dulbecco's Modified Eagle Medium (DMEM, Cambrex BioScience) supplemented with 20% fetal calf serum (FCS, GIBCO). E cells, dissociated enzymatically and mechanically, using trypsin and pipetting, were plated in ultra-low attachment flasks (Corning Life Sciences) at $6x10^4$ viable cells/ml in mammosphere medium, serum-free DMEM-F12 medium (Cambrex BioScience) supplemented with 20 ng/ml basic-fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin, and 0.4% bovine serum albumin (BSA), all from Sigma-Aldrich, as described (19, 29). Non-adherent spherical clusters of cells, named P1, were collected by gentle centrifugation after 7 days (d) and disaggregated through enzymatic and mechanical dissociation. The cells were analyzed microscopically for single-cellularity and seeded again at $6x10^4$ viable cells/ml to generate non-adherent spherical clusters of cells, named P2, and the process was repeated a third time to generate P3.

For confocal microscopy analysis, P1 after 2, 10 and 20 d of culture were centrifuged by cytospin on microscope slides and fixed in 3,5% paraformaldehyde (Sigma-Aldrich). Confocal analysis was performed with a

confocal laser scanning microscopy system (LSM510, Zeiss, Germany). All the images (512x512 matrix size) were acquired by using the same exposure time and brightness/contrast setting.

Clonal mammosphere formation assay

E cells were plated at 1 cell/well in 96-well plates or in 96-well ultra-low attachment plates, containing respectively 150 µl of epithelial or mammosphere medium. The number of E or P1 clones generated after 7 d of culture was evaluated and reported as number of clones generated every 100 single cells seeded. This procedure was repeated for P2 and P3, seeding a single mammosphere-derived cell/well.

Flow cytometry (FACS)

E, P1, P2, and P3 cells were collected after 7 d of culture and disaggregated through enzymatic and mechanical dissociation. They were then washed in PBS (Sigma-Aldrich) supplemented with 0.2% BSA and 0.01% sodium azide (Sigma-Aldrich) and either stained for membrane antigens or fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and then stained for intracellular antigens. The following antibodies (Ab) were used: Phycoerythrin (PE)/Cyanine (Cy)7-conjugated anti-CD24, rat Fluorescein isothiocyanate (FITC)-conjugated anti-CD29, Alexa Fluor647-conjugated anti–Sca-1, allophycocyanin (APC)/Alexa Fluor780-conjugated anti-Thy1.1, and Alexa Fluor647-conjugated anti-CD49f rat monoclonal Ab (mAb) from Biolegend;

purified anti-Musashi-1, anti-Octamer (OCT)-4, and anti-Cytokeratin (CK) 19 rabbit Ab, purified anti-CK18 and FITC-conjugated anti-CK14 and α-Smooth Muscle Actin (SMA) mouse Ab from Abcam; FITC-conjugated anti-H-2K^d from BD Biosciences; PE-conjugated rabbit anti-mouse Ig and FITC-conjugated goat anti-rabbit Ig from DakoCytomation. Samples were collected and analyzed with a CyAn ADP Flow Cytometer and Summit 4.3 software (DakoCytomation).

ALDEFLUOR assay

To evaluate aldehyde dehydrogenase (ALDH) activity, the ALDEFLUOR assay was performed according to the manufacturer's instructions (Stem Cell Technologies). Briefly, E, P1, P2 and P3 cells were collected after 7 d of culture and disaggregated, then suspended in assay buffer at 10^6 cells/ml and incubated with 5 µl/ml of activated BAAA (BODIPY-aminoacetaldehyde) substrate for 45 min at 37°C. Cells were then suspended in assay buffer and assayed on a CyAn ADP Flow Cytometer. Baseline fluorescence was established on control cells treated with BAAA and 10 µM Diethylaminobenzaldehyde (DEAB), a potent inhibitor of ALDH.

SASP effects on mammosphere formation

E cells were cultured at the density of $6x10^4$ cells/ml in a six-well dish in mammosphere medium in the presence or absence of SASP (0.1 and 0.5 mM, Sigma-Aldrich)or dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The total number of mammospheres for each well was counted after 7 d of culture and reported as number of mammospheres generated every $1x10^3$ E cells plated.

In Vivo Tumorigenesis Assay

E cells and P3 were collected after 7 d of culture, disaggregated, and implanted subcutaneously (s.c.) (200 and then 1000 cells) in the left flanks of BALB/c mice

(Charles River Laboratories). The growth of the tumor was monitored with calipers every week and reported as mean tumor diameter. Tumor-bearing mice were killed when the tumor exceeded 10 mm in mean diameter. Experiments were approved by the University of Turin institutional animal care and use committee.

In vivo treatment with SASP

BALB/c mice were injected i.v. with $5x10^4$ E or P1 cells. Three d after challenge, mice received intraperitoneally (i.p). two daily injection of saline (control) or 4 mg SASP (from a solution prepared immediately before each injection) until d 20, when mice were killed and the lungs removed and fixed in Formaldehyde solution (4%) for 4 d, cut into small pieces, paraffin-embedded, sectioned, and haematoxylin and eosin stained. Micrometastases were counted on six of more sections with a Nikon SMZ1000 stereomicroscope.

Microarray analysis

Quality and quantity of total RNA from samples were determined with an Agilent 2100 Bioanalyzer. cRNA synthesis, MouseWG-6 v2.0 Expression BeadChip hybridization, washing, and staining were performed as suggested by the manufacturers. Arrays were scanned on Illumina BeadStation 500. All arrays data were deposited in the GEO database, GSE21451.

Microarray Data Analysis

Probe average intensity signal was calculated with BeadStudio without background correction. Raw data were analyzed with Bioconductor using the oneChannelGUI package (30). Average probe intensities were log_2-transformed and normalized by the lowess method (31). All experimental groups were filtered to have an inter-quartile range for each probe ≥ 0.25 . Differential

expression between E and P1, P2 or P3, was assessed by using an empirical bayes method (32) together with a false discovery rate (FDR) correction of the P-value (33).

The PAMR method (34) was used to evaluate the efficacy of the red cluster transcripts to discriminate between CIC and not-CIC transcription profiles. Cho, Liu and Charafe-Jauffret Affymetrix CEL files were retrieved as indicated in their papers (16, 17, 35). Probeset intensities were obtained for each data set by means of Robust Multichip Average and normalization was done according to the quantiles method (31). Only the probesets linked to Entrez Gene IDs of the red cluster transcripts were considered for further analysis.

The target selection procedure consists of two steps: ranking on the basis of clinical outcome (CO score) and ranking on the basis of expression in normal tissues (NOR score).

CO score: intensity data, for a putative target, are extracted from a cancer data set together with the clinical variable of interest, i.e. distant metastasis outcome. K-mean clustering (K=2) is used to divide the specimens in two groups, i.e. up-regulated and down-regulated. For the two groups an exponential survival regression model is fitted and a p-value for the separation between the up-regulated/down-regulated survival curves is calculated (P_{TRUE}). The P_{TRUE} is used in equation (i, ii) together with a null distribution (P*) constructed randomizing samples outcomes (n = 10000 times) to generate the CO score. If the exponential survival regression model for the up-regulated group is associated with a poor outcome:

$$COscore = \log_{10}\left(\frac{1}{n}\sum I(P_{TRUE} \geq P^{*})\right) \quad \text{(i)}$$

If the exponential survival regression model for the down-regulated group is associated to a good outcome:

$$COscore = -\log_{10}\left(\frac{1}{n}\sum I(P_{TRUE} \geq P^{*})\right) \quad \text{(ii)}$$

The final CO score is given by the mean of the CO scores calculated over the various data sets taken in consideration. i.e. GSE2034, GSE1456, GSE2990, GSE3494, GSE6532, GSE7390, GSE11121.

NOR score: For each normal tissue the rank product (iii)(36) is calculated for the putative target under investigation and for each of the four ERB family members (EGFR, ERBB2-4). k is the number of replicates of a specific tissue in the normal tissue dataset and r_i the rank of a gene in the i-th replicate.

$$RP = \left(\prod_{i=1}^{k} r_i\right)^{1/k} \quad \text{(iii)}$$

A NOR score is then calculated (iv). n is the number of normal tissue types present in the normal tissue microarray dataset represented by the Body Index data set (GSE7307, 512 arrays representing 82 adult tissues):

$$NOR\ score = \log_{10}\left(\frac{1}{n}\sum_{i=1}^{n} I(RP_{OA} \geq RP_{ERB})\right) \quad \text{(iv)}$$

CO score and NOR score were ranked using TOPSIS, a Multi-criteria Decision making method (37), to detect the top ranking targets, i.e. those having the lowest combined CO/NOR score.

Statistical analysis

Differences in tumor latency were evaluated with the Mann-Whitney test, those in tumor protection by a contingency test (χ^2), and those in clonal sphere formation, cytokine production, marker expression and number of metastasis with Student's t test.

Results

Mammosphere generation

After 2 d of culture of TUBO cells in non-adherent conditions (19, 29) floating spherical mammospheres took shape (P1). After 10 d, they were symmetrically encapsulated to form ‘‘golf ball’’ that became hollow around the third week and remained the same size (Figure 1A). These mammospheres were dissociated every week, and serially propagated at clonal density in secondary (P2) and tertiary (P3) passages.

To assess the self-renewal of these mammospheres, TUBO cells and cells dissociated from P1 and P2 were cloned in limiting dilution by plating one cell/well in mammosphere medium. TUBO cells were also cloned in limiting dilution in adherent conditions to generate epithelial E clones. The number of E, and P1 to P3 clones after one week of culture increased progressively from E- to P3-derived cells (Figure 1B) as evidence of both the presence and the progressive enrichment of self-renewing cells.

Tumor-initiating ability of mammospheres.

A s.c. challenge of syngeneic BALB/c mice with 2 x 10^2 E-cells produced no tumors, whereas P3-derived cells formed tumors in all mice (Figure 1C). Challenges of 1 x 10^3 P3-derived cells gave rise to fast growing tumors in all mice, whereas a similar challenge of E-derived cells gave rise to tumors in 4/6

mice, but only two tumors reached a 10 mm mean diameter in the next 100 days (Figure 1D).

Stem-cell markers and MHC I expression on mammosphere-derived cells.

We had shown that CIC from primary BALB-neuT mammary carcinomas express the Sca-1 (19). Present data show that Sca-1 is not expressed on E cells, whereas its expression progressively increases in those derived from P1 to P3 (Figure 2A). A similar induction of the expression of Musashi-1 (38) and Thy1.1 (35) antigens was found (Figure 2B, C). Moreover, P1 to P3-derived cells, but not E cells, were positive for OCT-4 (Figure 2D), a stem-cell marker of breast CIC (19, 29). Additional evidence for the CIC enrichment was provided by progressive enhancement of ALDH activity (16) from E- to P1- to P3-derived cells (Figure 2F). On the other hand, expression of the mouse MHC class I molecule H-2K^{d} drastically decreased from E cells to P1-P3-derived cells (Figure 2E) in keeping with the ability of CIC to evade immune control (39).

No significant changes in the expression of CD24, CD29, CD49fm, CK18 and CK19 were found. Expression of CK14 increased in the passage from E- to P1-derived cells, but then decreased in P2- to P3-derived cells. By contrast, α-SMA displayed a slight progressive decrease from E- to P1-P3-derived cells (Supplemental Table 1).

Transcription profiling

The transcription profiling of E-cells was compared with that of P1, P2 and P3-derived cells in three independent experiments using MouseWG-6 v2.0 Illumina beadchips (GSE21451). Following normalization and removal of those not expressed and not changing, the differential gene expression was assessed with the regularized *t*-test by comparing P1, P2 and P3 derived cells with E-cells using a FDR ≤ 0.05 (33), together with an absolute $\log_2$ fold change threshold ≥1. The union of transcripts differentially expressed in E versus P1, P2 and P3-derived cells produced a total of 495 transcripts (Figure 3A, Supplemental Table 2) whose expression was then shown as $\log_2$ ratio between the intensity averages of mammosphere-derived cells with respect to E-cells, and analyzed by K-mean clustering (Figure 3B). This analysis disclosed both a cluster of transcripts whose expression was rising (Figure 3B, red cluster), and three clusters (Figure 3B, blue, yellow and green clusters) whose expression was decreasing from E to P1-P3-derived cells.

As mammosphere medium contains EGF, insulin and bFGF that may influence transcript expression, we used the IPA 7.0 (www.ingenuity.com) software to look for direct and indirect interactions between the 495 transcripts differentially expressed and these three growth factors. The 35 genes clustered by this analysis were removed from the data set (not shown).

Red cluster genes discriminate CIC from non-CIC transcription profiles.

To identify transcripts associated with CIC, we focused on the red cluster. This comprised 183 genes following the removal of those linked to EGF, insulin and bFGF (Supplemental Table 2). We then used three public breast cancer data sets (16, 35, 40) to investigate the molecular characterization of cancer stem cells, and determine whether expression of the red cluster genes provides a signature discriminating CIC from not-CIC transcription profiles (16, 35, 40).

Cho et al. (35) have shown that mouse Thy1.1^{+} CD24^{+} CD45^{-} breast tumor cells have higher tumor initiating ability than "Non-Thy1.1^{+}CD24^{+}". By using Affymetrix GeneChips (GSE8828), they detected a transcription signature whose association with survival was assessed in cancer patients. We mapped our red cluster genes on the corresponding Affymetrix probesets via Entrez Gene Identifiers (41). The 149 Entrez Gene Identifiers present in both the red cluster and in Cho's et al's data set discriminate between Thy1.1^{+}CD24^{+} and "Not Thy1.1^{+}CD24^{+}" cells by means of the shrunken centroid method (34) (Table 1A). Furthermore, we found that the Thy1.1^{+} cell fraction increases from P1 to P3-derived cells (Figure 2B).

A similar approach was also applied to the data set of Liu et al's comparison (40) of the gene-expression profile of human CD44^{+} CD24$^{-/low}$ breast cancer cells with a CIC phenotype with that of normal breast epithelium. They used the differentially expressed genes (CIC versus normal breast) to generate a gene

signature of invasiveness, whose association with survival was then assessed in cancer patients. Here, too, our red cluster genes discriminated $CD44^{+}CD24^{-/low}$ tumorigenic breast cancer cells from normal breast epithelium (Table 1B).

Lastly, in Charafe-Jauffret et al's paper (16), human breast cancer $ALDEFLUOR^{+}$ cells characterized by a distinct molecular profile and CIC properties were isolated and a gene signature discriminating $ALDEFLUOR^{+}$ versus $ALDEFLUOR^{-}$ cells was defined. Similar discrimination, albeit with a larger classification error (not shown), was also displayed by our red cluster genes (Table 1C), and the $ALDEFLUOR^{+}$ fraction increased from P1 to P3-derived cells (Figure 2F).

Taken together, these results show that the red cluster genes constitute a transcription signature for breast cancer CIC.

Oncoantigens among red cluster genes.

To select ideal oncoantigens with explicit oncogenic potential and limited expression in normal tissues (2, 21) we devised a ranking procedure based on two scores: CO and NOR (see Material and Methods) (21). CO score assessed on seven human breast cancer datasets (1500 arrays, GSE2034, GSE1456, GSE2990, GSE3494, GSE6532, GSE7390, GSE11121, Supplemental Table 3) ranked the red cluster genes in function of the ability of a tumor to metastasize (Figure 4 and Supplemental Table 2). On the other hand, since in a microarray analysis absolute expression values defy quantification, the expression of red cluster

genes in normal tissues was assessed in comparison with reference genes (21). The NOR score (Supplemental Table 2) was thus calculated by comparing the expression of red cluster genes with that of genes of the ERB family within the Body Index data set (GSE7307). The two scores were used to rank genes with the TOPSIS algorithm (37), a method based on the simple concept that the final ranking is structured in such a way that the highest rank has a minimum distance from the ideal situation, and a maximum distance from the negative-ideal one, i.e. the solution which characterizes the best target. The ideal solution is formed as a composite of the best performance values (normalized to a-dimensional measures) displayed by any alternative for each score; the negative-ideal solution is the composite of the worst performance values. Proximity to each of these performance poles is measured in the Euclidean sense, with equal weighting of each score.

On looking for good targets for both antibody and cytotoxic cells, we first focused on genes whose products are expressed on the cell membrane (2, 21). Two of the red cluster gene products located on cell surface were ranked in the first 1/3rd of the best CO and NOR scores (Supplemental Table 2): TMPRSS4 (Figure 4, red arrow, Supplemental Table 2, bold red), a membrane linked serine protease, and xCT (SLC7A11, Figure 4, blue arrow, Supplemental Table 2, bold blue), the functional subunit of the cystine/glutamate transporter xc-system.

The expression on the cell surface of P1, P2 and P3-derived cells was then confirmed by FACS analysis (Supplemental Figure 1).

Red cluster genes and drug response

These two top-scoring oncoantigens are presumed to be good vaccination targets. The literature also shows that they are down-modulated by two drugs: SASP disrupts xCT's functional efficiency (42) and impairs the metastatic potential of xCT^+ cells (27), while the tripeptide tiroserleutide, not yet commercially available, indirectly down-modulates TMPRSS4 expression (43). We observed that SASP impairs the formation of mammospheres (Figure 5A), and also inhibits lung metastases that follow an i.v. challenge of P1-derived cells more efficiently than those formed by TUBO cells (Figure 5B).

Interrogation of the Ingenuity Knowledge revealed that Atorvastatin, a cholesterol lowering drug with antileukemic effects (44), is linked to four red cluster genes coding for intracellular proteins (Supplemental Figure 2A). In the absence of information on the relation between these genes and CIC, we looked to see whether Atorvastatin affects the ability of TUBO cells to grow and form mammospheres. Its addition to the culture medium does not impair E-derived cell proliferation, but the number of mammospheres produced (Supplemental Figure 2B). These results suggest that selective CIC inhibition may be another feature of this multifunctional molecule.

Discussion

In this paper we report that successive passages of mammospheres derived from the ERBB2^{+} mouse TUBO mammary carcinoma cell line display a progressive enrichment of self-renewing cells with stem cell markers and enhanced tumor-initiating ability. Their genome-wide transcription profiling allowed us to cluster a set of mouse transcripts whose expression raises passing from TUBO cells grown as epithelial monolayers to the cells of the successive mammosphere passages. Following the removal of transcripts whose differential expression was associated with growth factors present in the mammosphere culture medium, the remaining transcripts lead us to identification of 183 mouse genes (the red cluster genes) that provide a signature distinctive of the subpopulation of TUBO cells retaining CIC characteristics. However, mouse genes defined in an experimental system are primarily of interest if they have a human orthologue with a similar role, and are not merely idiosyncratic (21). Validation of the red cluster gene signature by meta-analysis of three public data sets profiling human stem-like cancer cells (16, 35, 40) proved that they provide a transcription signature for mouse and human breast cancer CIC.

The red cluster gene signature was therefore investigated in search for potential new oncoantigens expressed by CIC. Oncoantigens have so far been searched for in invasive tumors (2, 21, 45). Identification of those expressed by CIC could

provide an unprecedented opportunity for targeting molecules delivering signals crucial for cancer stemness. This is a crucial issue in order to devise new and more effective anti-tumor vaccines. As the stem cell properties of CIC make them responsible for tumor progression, metastases and recurrences, it is conceivable that identification of oncoantigens involved in the maintenance of their stem-cell like features could extend the efficacy of anti-cancer vaccination.

An ideal oncoantigen targeted by a vaccine should display a low level of expression in normal tissues and high level in CIC or in their microenvironment (21). To identify the red cluster genes coding for potential oncoantigen targets for anti-tumor vaccination, we devised a ranking procedure based on both the relationship between red cluster genes and the clinical outcome (the CO score) and their low expression in normal tissue (the NOR score). In addition we first selected oncoantigens expressed on the cell surface, that would be the target of both the cell-mediated and the antibody-mediate immune response (2). Since antibodies do not require MHC molecules in order to recognize and bind their target, their activity is not impaired by the down-modulation of MHC molecules on the surface of tumors cells (21).

The two most promising CIC putative oncoantigens (i.e. those with the best CO and NOR scores) expressed on the cell membrane were TMPRSS4 and xCT. TMPRSS4 seems to be an ideal target for vaccination since it is a type II transmembrane serine protease with an extended extracellular proteolytic

domain (46-48) that recently has been shown to be associated to an Epithelial-mesenchymal transition (EMT) signature or breast cancer cells (49). xCT is a 12 pass transmembrane heteromeric amino acid transporter eliciting sodium-independent exchange of anionic L-cysteine and L-glutamate (22). Its functional efficiency is inhibited by the anti-inflammatory molecule SASP, and this inhibition greatly reduces the metastatic potential of the expressing tumor cells (27). The potential role of xCT as oncoantigen is endorsed by our data of the ability of SASP to impair the formation of mammospheres. The ability of SASP to inihibit lung metastasis formed by mammosphere cells further proves the involvement of xCT in CIC functionality. Although in our model system SASP does not show any specific effect on non-CIC cells as described previously (27).

Notably, both TMPRSS4 and xCT participate in down-regulating E-cadherin expression (25, 27). E-cadherin has a potent malignancy-suppressing activity and its functional inactivation induces epithelial-mesenchymal transition, a key developmental program often activated during cancer invasion and metastasis, in which epithelial cells gradually lose their epithelial structures while concomitantly acquiring the characteristics of mesenchymal cells (50).

Lastly, interrogation of red cluster genes may provide unforeseen information also on the effect on CIC of pharmaceutical products not specifically designed to treat cancer. An example is given by Atorvastatin, which was spurred by the data provided by Ingenuity Knowledge based analysis linking it to four of the red

cluster genes. Our experiments showed that this cholesterol lowering drug with antileukemic effects *in vitro* and *in vivo* (44), drastically inhibits the expansion of mammosphere-derived cells.

In conclusion, a transcriptional characterization of mammary CIC from a mouse mammary cancer cell line and meta-analyses of independent human breast tumor data sets have led to the identification of a gene signature of CIC that smoothly applies to human mammary carcinomas. Interrogation of this signature provides fresh indications on new potential oncoantigens and interaction of CIC with pharmaceutical compounds and underlines the role of xCT in CIC maintenance *in vitro* and *in vivo*.

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Figure Legends

Figure 1: Mammosphere generated from TUBO cells have self-renewal and tumor-initiating ability. A) Representative images of the morphological analysis of mammospheres generated from TUBO cells growing in non-adherent conditions are shown. After 2 d, cell spheroids are visible. After 10 d, they are symmetrically encapsulated to form ‘‘golf balls’’ that become hollow around the third wk. Bar, 100 μm. B) Ability of E- and P1- to P3-derived cells to produce mammospheres in a limiting dilution assay. Self-renewal ability increases from E- to P3-derived cells. Data are means ± SD of four independent experiments ($P<0.05$). C, D) Tumor initiating ability of E (dotted line) and P3-derived cells

(continuous line) measured by the ability of 200 (C) and 1000 (D) cells to produce subcutaneous tumor in syngeneic mice. For all conditions n=6.

Figure 2: Modulation of the expression of stem cell markers and MHC I glycoproteins on E cells and mammosphere-derived cells. After dissociation, E, P1, P2 and P3 cells, collected after 7 days of culture, were stained for Sca-1 (A), Musashi-1 (B), Thy1.1 (C), OCT (D), H-2K^d (E) antigen and for ALDH activity (F). Data represents the percentage of positive cells of at least three independent experiments; horizontal lines represent means.

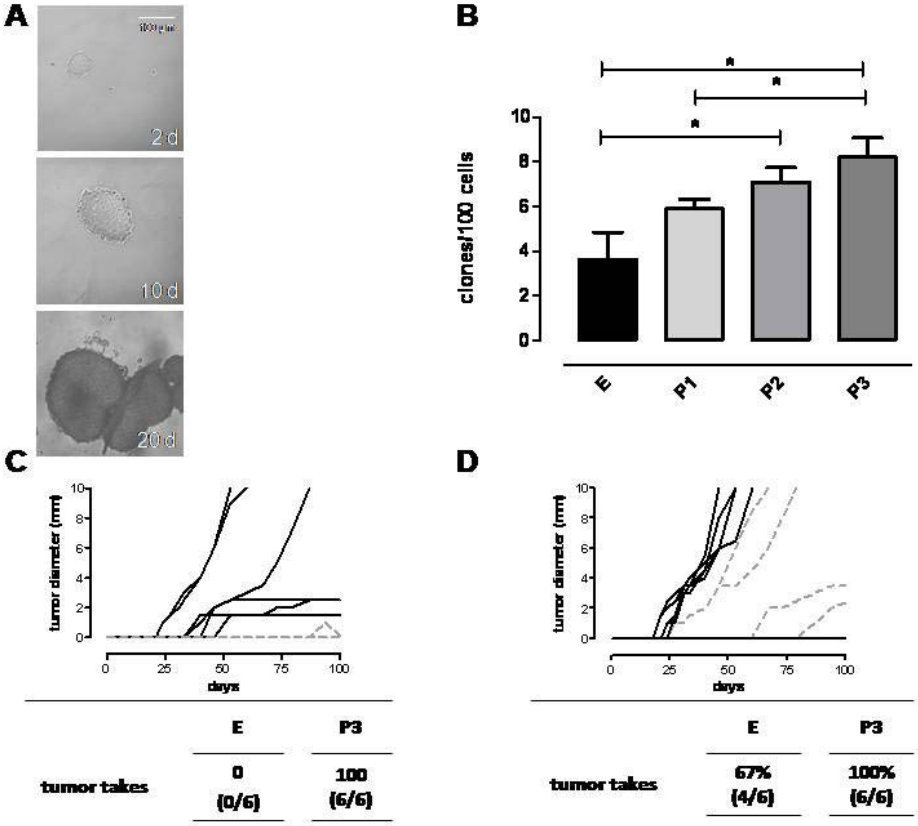
Figure 3: Genome-wide transcription profiling and hierarchical clustering depicted a transcript signature whose expression raises passing from E to P1-P3. A) Venn diagram of the three set of differentially expressed genes found by comparing P1-P3 with respect to E cells. B) K-mean clustering, K=4, of average ratio between P1-P3-cell derived gene expression with respect to E-cells. Clustering was done on the 495 transcripts given by combination of the three comparisons showed in A. The transcript signature rising in expression from E to P1-P3 is shown in red (red cluster).

Figure 4: Ranking red cluster genes on the basis of clinical outcome (CO) of tumors expressing them and their low expression on normal tissues (NOR)

allowed the identification of two targets associated with CIC: xCT and TMPRSS4. Red cluster genes colored on the basis of their CO score. CO score was assessed on seven human breast cancer datasets (1500 arrays) ranking the red cluster genes in function of the ability of a tumor to metastasize. Green color genes are those whose up-regulation is associated with a poor outcome. The NOR score was calculated by comparing the expression of red cluster genes with that of genes of the ERB family within the Body Index data set (GSE7307). The two scores were used to rank genes within the framework of the Multi-Criteria Decision Making models. Red and blue arrows indicate the two genes with top-ranking CO and NOR scores located in the plasma membrane compartment. Uncolored genes are some of the stemness/differentiation markers measured in our study and connectable, via TRADD and CASP8 (yellow), to some of the red cluster genes.

Figure 5: SASP impairs mammospheres self-renewal and metastasis formation. A) The effect of SASP on P1 mammosphere formation (grey bar) was compared with those obtained after incubation with DMSO (white bar) or medium alone (black bar). B) Effect of SASP (closed circles) or saline (open circles) on the ability of an i.v. challenge of E- or P1-derived cells to form lung metastases. A graph representing two independent experiments is shown. Each circle represents the number of micrometastasis identified in three or more sections.

Figure 1



	E	P3
tumor takes	0 (0/6)	100 (6/6)

	E	P3
tumor takes	67% (4/6)	100% (6/6)

Figure 2

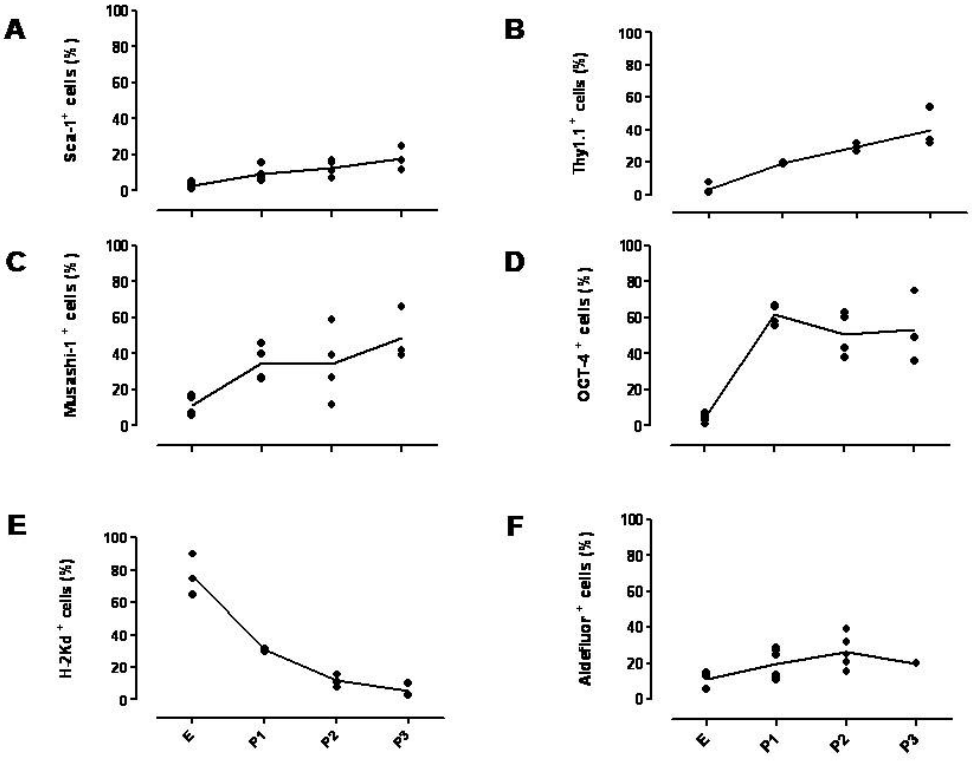

A
Sca-1+ cells (%)
B
Thy1.1+ cells (%)
C
Musashi-1+ cells (%)
D
OCT-4+ cells (%)
E
H-2Kd+ cells (%)
F
Aldefluor+ cells (%)
0
20
40
60
80
100
E
P1
P2
P3

Figure 3

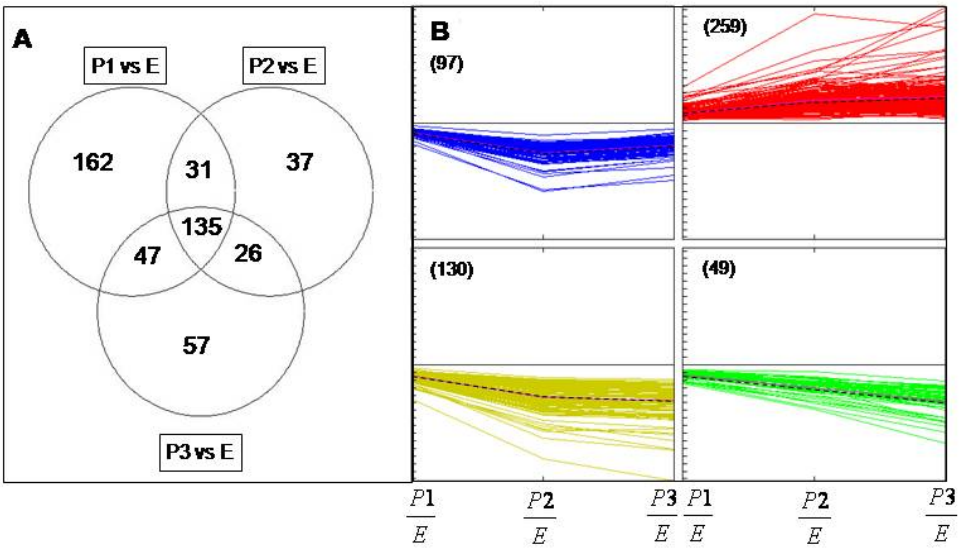
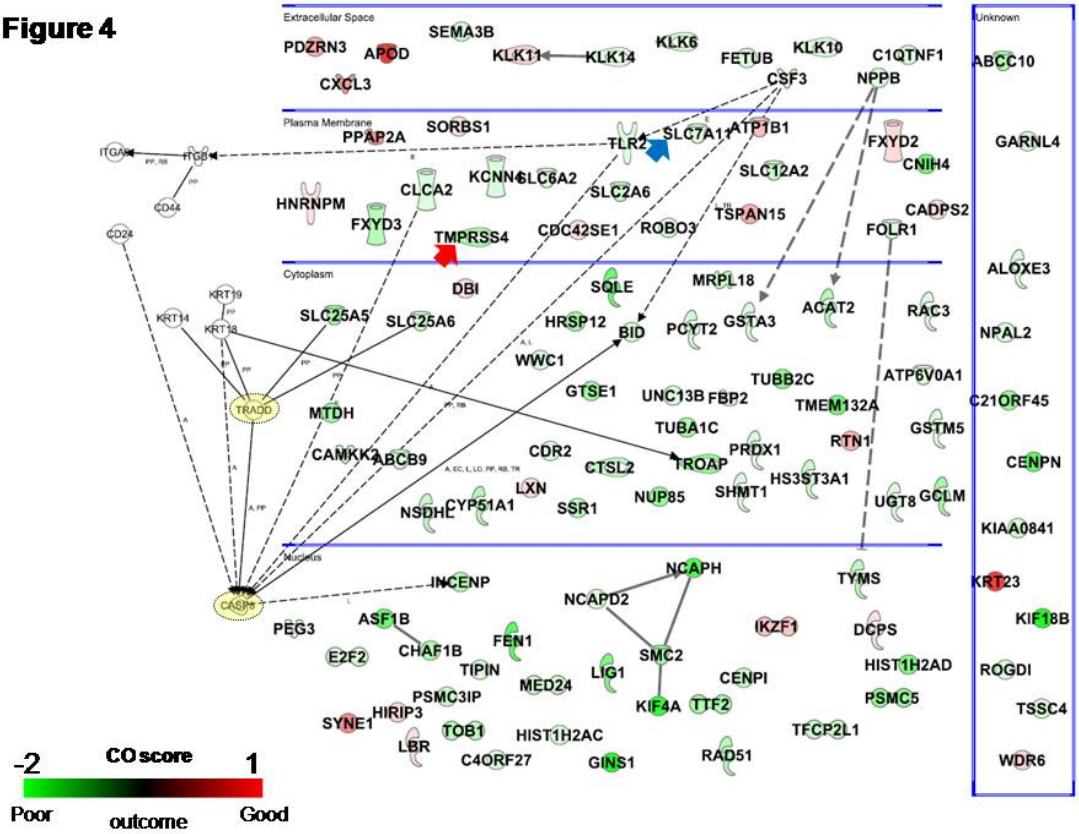

A
P1 vs E
P2 vs E
P3 vs E
162
31
37
135
47
26
57
B
(97)
(259)
(130)
(49)
P1/E
P2/E
P3/E
P1/E
P2/E
P3/E

Figure 4


Extracellular Space
Plasma Membrane
Cytoplasm
Nucleus
Unknown
PDZRN3
APOD
SEMA3B
KLK11
KLK14
KLK6
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KLK10
C1QTNF1
CXCL3
CSF3
NPPB
ABCC10
SORBS1
PPAP2A
TLR2
SLC7A11
ATP1B1
FXYD2
GARNL4
CNIH4
SLC12A2
CLCA2
KCNN4
SLC6A2
SLC2A6
HNRNPM
FXYD3
TSPAN15
CADPS2
FOLR1
CDC42SE1
ROBO3
TMPRSS4
ALOXE3
DBI
SQLE
MRPL18
ACAT2
RAC3
SLC25A5
SLC25A6
HRSP12
BID
PCYT2
GSTA3
NPAL2
WWC1
TUBB2C
ATP6V0A1
GTSE1
UNC13B
FBP2
TMEM132A
C21ORF45
TRADD
MTDH
TUBA1C
GSTM5
CAMKK2
ABCB9
CDR2
PRDX1
RTN1
CENPN
TROAP
CTSL2
HS3ST3A1
LXN
NUP85
SHMT1
UGT8
GCLM
NSDHL
CYP51A1
SSR1
KIAA0841
NCAPH
TYMS
INCENP
KRT23
NCAPD2
CASP8
PEG3
ASF1B
IKZF1
DCPS
KIF18B
FEN1
SMC2
E2F2
CHAF1B
HIST1H2AD
TIPIN
LIG1
CENPI
ROGDI
MED24
PSMC3IP
KIF4A
TTF2
PSMC5
HIRIP3
SYNE1
TSSC4
TOB1
HIST1H2AC
TFCP2L1
LBR
C4ORF27
GINS1
RAD51
WDR6
-2
CO score
1
Poor
outcome
Good

Table 1

Ability of red cluster transcripts to discriminate CIC cells from non-CIC cells

A		Expected	
		Thy1$^+$CD24$^+$	Not Thy1$^+$CD24$^+$
Detected	Thy1$^+$CD24$^+$	3	1
	Non-Thy1$^+$CD24$^+$	0	2
Error: 0.16			

B		CD44$^+$CD24$^{-/low}$	normal breast epithelium
Detected	CD44$^+$CD24$^{-/low}$	3	0
	normal breast epithelium	0	3
Error: 0			

C		ALDEFLUOR$^+$	ALDEFLUOR$^-$
Detected	**ALDEFLUOR$^+$**	5	2
	ALDEFLUOR$^-$	3	6
Error: 0.312			

Red cluster transcripts were detected in three public available data set comparing CIC versus non-CIC transcription profiles: (A) Cho et al. (ref. 35); (B) Liu et al. (ref.40); (C) Charafe-Jauffret et al. (ref. 16). The PAMR classifier was used to evaluate the efficacy of red cluster transcripts to discriminate CIC versus non-CIC transcription profiles. Leave One-Out Cross-Validation error is shown for each data set.

Supplemental Methods

Atorvastatin effects on mammosphere formation

E cells were cultured at the density of $6x10^4$ cells/ml in a six-well dish in mammosphere medium in the presence or absence of Atorvastatin (2 µM, Sigma-Aldrich) or DMSO (Sigma-Aldrich). The total number of mammospheres for each well was counted after 7 d of culture and reported as number of mammospheres generated every $1x10^3$ E cells plated.

Flow cytometry of TMPRSS4 and xCT expression

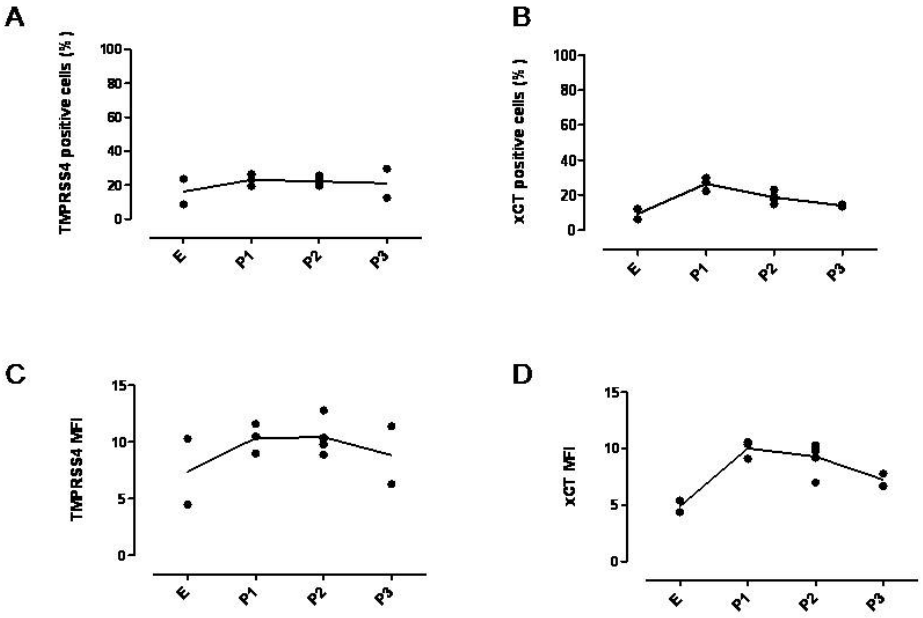
E, P1, P2, and P3 cells were collected after 7 d of culture and disaggregated through enzymatic and mechanical dissociation. They were then washed in PBS (Sigma-Aldrich) supplemented with 0.2% BSA and 0.01% sodium azide (Sigma-Aldrich) and fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). Cells were then incubated for 5 min at room temperature with goat serum (Sigma-Aldrich) and then for 30 min at 4°C with either goat anti-TMPRSS or goat anti-xCT Abs (Santa Cruz Biotechnology). After two washes in wash buffer (BD Biosciences) cells were incubated 30 min at 4°C with FITC-conjugated rabbit anti-goat immunoglobulins (DakoCytomation). After two additional washes, samples were collected and analyzed with a CyAn ADP Flow Cytometer and Summit 4.3 software (DakoCytomation). Cells were gated according to their light-scattering properties to exclude cell debris.

Caption to supplemental figures

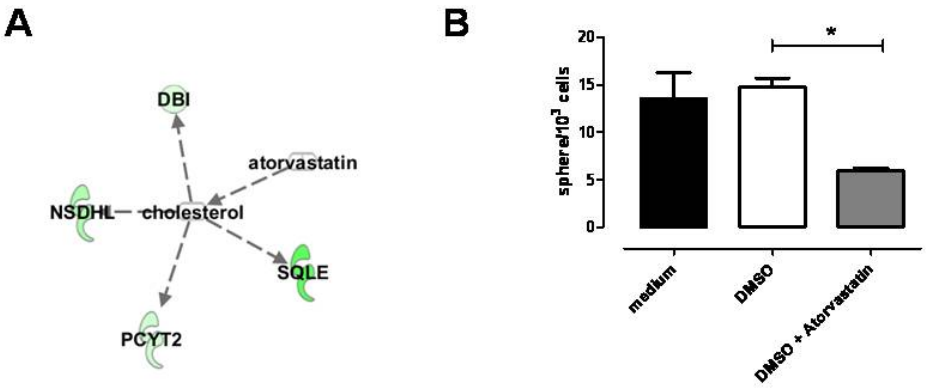
Supplemental Figure 1. Expression of TMPRSS4 and xCT in E cells and mammosphere-derived cells. After dissociation, E, P1, P2 and P3 cells, collected after 7 d of culture, were stained for TMPRSS4 (A, C) and xCT (B, D) and analyzed by flow cytometry. Data represent the percentage of positive cells (A, B) and the mean fluorescence intensity (MFI, C, D) of at least three independent experiments.

Supplemental Figure 2: Atorvastatin effect on mammosphere formation. A) Genes found associated to Atorvastatin by Ingenuity knowledge based analysis. DBI (Diazepam-binding inhibitor), SQLE (squalene epoxidase), PCYT2 (phosphate cytidylyltransferase 2, ethanolamine) and NSDHL (NAD(P) dependent steroid dehydrogenase-like). B) To evaluate the effect of atorvastatin on P1 mammosphere formation, E cells were cultured at the density of $6x10^4$ cells/ml in a six-well dish in mammosphere medium (black bar) in the presence or absence of Atorvastatin (grey bar,) or DMSO (white bar). The total number of mammospheres for each well was counted after 7 d of culture and reported as number of mammospheres generated every $1x10^3$ E cells plated. Results are shown as mean ± SD of three independent experiments. * $P<0.05$, Student's t test.

Supplemental Figure 1



Supplemental Figure 2



DISCUSSION

The main feature of statins is the ability to inhibit the enzyme HMG-CoA thereby generating the inhibition of cholesterol, mevalonate and isoprenoids synthesis in mammalian cells. . Cholesterol is essential in maintaining cellular membrane structure and integrity, and it also serves as a precursor for the synthesis of isoprenoid (4). The most relevant studies indicated that the prenylation of other proteins, including the Ras and Rho family proteins, is the crucial step in the apoptotic, angiogenic and inflammatory effects of statins. In vitro studies on various cell lines have shown that statins have potentials growth inhibitory (29-30).

. For this reason statins may be considered as potential adjuvant drugs in anticancer therapy. However few and conflicting data exist about the efficacy of statins in preventing tumor onset on animal models and their efficacy in clinical trials, although recent works have shown that the HMG-CoA inhibitors prevents the onset of estrogen-receptor negative tumors and reduced the proliferation of the most aggressive isotypes of breast cancers.

Virgin female BALB-neuT provide one of the most aggressive models of mammary carcinogenesis since the product of the rHer-2 oncogene is already over-expressed on the cell surface of the rudimentary mammary glands of 3-week-old females that inevitably develop lethal metastasizing carcinomas in all ten mammary glands by week 33. These tumors are highly positive for Her2 expression (86)

The subgroup of Her2 overexpressing breast cancers is a particularly interesting model to evaluate the statins effects, because one of the most active downstream transducer of the Her2 oncogene is Ras GTPase, whose activity is strictly dependent on the mevalonate pathway.

In the first part of this study, we observed the effects exerted by atorvatatin administration on BALB-neuT mice. The BALB-neuT mice were daily treated with

12µg/day of atorvastatin a dose roughly equivalent to 40mg/kg/day used as anti- colesterolemic therapy in patients. In this mouse model there is the possibility to monitorate the tumor progression directly in mammary glands, and in a physiological condition.

We also investigate the effect of atorvastatin on reduction of isoprenoids synthesis and the amount of ubiquinone, in epithelial TUBO cells line, derived from Her2 overexpressing transformed cells of BALB-neuT mammary tumors.

Atorvastatin effectively inhibited its natural target HMGCoA reductase, thus decreasing the rate of several metabolite produced in mevalonate pathway like the FPP, IPP, the end- product, cholesterol and ubiquinone. The decrease of ubiquinone synthesis leads us to suppose that TUBO cells statin treated have a lowed supply of ATP. Indeed, the presence of ubiquinone is essential for the efficient activity of respiratory of mitochondrial chain. Atorvastatin decreased the levels of FPP, generates the reduction of Ras activity, dawn stream effector of HER2 costitutive active in TUBO cells.

The expression of total Ras did not change between BALB/c and BALB-neuT mice, but there are important difference in the active amount of GTP-Ras protein, indeed in BALB-neuT mice treated with atorvastatin the activation of Ras was prevented.

In parallel, by limiting the cell amount of isoprenoids, the drug reduced the activity of the geranylgeranylated RhoA protein, a second small GTPase present in TUBO cells and in tumor glands, and of its downstream effector RhoA kinase. Although RhoA is often overexpressed in breast cancers and is crucial for breast epithelium proliferation and invasion (87)]. Its activity did not change between BALB/c and BALB-neuT mice, suggesting that RhoA activation does not play a critical role in the transformation of cells driven by Her2 oncogene. The striking difference in Ras activity between wild-type and transgenic mice lead instead to

hypothesize that Ras has a prominent role in the tumorigenesis and/or in the tumor growth in BALB-neuT mice.

The activation of ERK1 and ERK2 kinases in our experimental model strictly follows the Ras activity : phospho-ERK1/2 were absent in BALB/c non transformed cells, present in BALBneuT tumor glands and in TUBO cells, and was markedly reduced by atorvastatin. On the other hand, the activity of the PI3K/Akt pathway, another downstream effector of Her2 receptor displayed minor changes in the presence of the statin: a small amount of phosphorylated active Akt was detectable also in non transformed mammary glands, which are devoid of constitutive active Her2. In addition we observed a slight increase of activated Akt in BALB-neuT mice which is slightly reduced by the atorvastatin administration. Akt may also be Ras-independent (86) that would explain the limited effect exerted by atorvastatin on the activity of this protein.

Therefore, the effect of atorvastatin on activity inhibition of Ras and ERK1 / 2 and the little reduction of Akt activation in mammary glands of BALB-neuT mice, are the right context which suggests a possible reduction of tumor growth in BALB-neuT mice.

Unexpectedly we did not observed any growth reduction of tumor cells implanted in statin-treated BALC/c mice, and similar effect is exerted in BALB-neuT mice were did not observe any delay on tumor onset.

NF-κB fulfills a central role in the cellular stress response and in inflammation by controlling the expression of a network of inducers and effectors that define responses to pathogens and other classes of danger signals as well as oxidative stress or drug. This molecule in normal conditions is retained in cytosol by the inhibitor Ikbα protein; on the contrary in conditions of cellular stress this molecule is phosphorylated and was dislodged from NF-kB allows translocation into the nucleus (88).

The relationship between statins and NFkB pathway is still a matter of debate and is highly variable depending on cell type.

Interestingly it has been reported that the activity of NF-kB can be reduced by isoprenoids derived from mevalonate pathway, such as FPP (89) and GGPP (90). In our experiment we observed that atorvastatin increased the nuclear translocation of NF-kB, by reducing the amount of the inhibitory protein IkBα. The statin effect was due to the inhibition of HMGCoA reductase, because it was abolished by mevalonate and by intermediate metabolites, like FPP and GGPP analogues. This observation suggest that the statins effect on NF-kB are likely due to the decrease of G-proteins activity.

Tumor cells are able to survive and proliferate even if proliferation signal are turned down. This feature allows to explain why the administration of atorvastatin therefore should be a good inhibitor of proliferation, decreasing the activity of Her2 effectors oncogene as well as Ras ERK and Akt; and result unable to inhibit tumor onset on BALB-neuT mice and TUBO cells proliferation.

Only when we inhibited the activity of NF-kB,statin can significantly reduce proliferation and increase apoptosis in Her2 positive cells.

In many works has recently been studied that stem cells may play a decisive role not only in the generation of complex multicellular organisms but also in the development and progression of tumors. The main features of these cells are the ability to self-renew and to cause the heterogeneous lineages of cancer, cell that comprise the tumor. .

First of all in this study we generate mammosphere form TUBO cells line and we test their ability to self-renewal. To confirm that cancer stem cells are able to generate a tumor P3 cells and E cells were s.c. challenge in BALB/c mice; the P3 cells have higher tumorigenic potential than bulk tumor cells E, that are unable to gave rise tumor.

Mammosphere, were characterized by the expression of some markers of stem cells, as well as Sca-1 whose expression was found in CIC derived from tumor of BALB-neuT mice (91) and other stemness markers not present on E cells but only on P1, P2 and P3 mammosphere. In particular the mammosphere and not E cells, were positive to OCT-4 a specific marker of mammary stem cells (91) (92). This result suggests that there are the possibility to find a genes differentially expressed between E cells and P1, P2 and P3 cells against which direct a potential anti-cancer therapy.

The first step was removal of transcripts whose differential expression was associated with growth factor present in mammosphere culture medium, the remaing transcript leads us to identification 183 mouse gene that provide a signature distinctive if the subpopulation of TUBO cells retaining CIC characteristic. Among these gene the two most promising CIC putative oncoantigene expressed on the cell membrane were TMPRSS4 and xCT.

xCT is a transmembrane heteromeric amino acid transporter eliciting sodium-independent exchange of anionic L-cysteine and L-glutamate (93). Its functional efficiency is inhibited by the anti-inflammatory molecule SASP, and this inhibition greatly reduces the metastatic potential of the expressing tumor cells (94). The role of CICs in multistage cancer progression, particularly with respect to metastasis, has not been well-defined. Cancer metastasis requires the seeding and successful colonization of specialized CICs at distant organs.

In our experimental condition the administration of SASP in vitro generates the inhibition on mammosphere formation. At the light of this date we show that SASP administration in BALB/c mice in vivo is able to inhibit lung metastasis formation after tail injection of mammosphere.

Furthermore, the additional analysis of data provided by Ingenuity, has generate a connection between the cluster of genes differentially expressed in mammosphere and atorvastatin.

A recent study of statins on mouse embryonic stem cells demonstrated that statins inhibited their cell proliferation and also led to loss of self-renewal capacity. This was mediated by RhoA geranylgeranyl inhibition, and the inhibitory effects were reversible with GGPP substitution (95)

Furthermore, both embryonic stem and cancer stem cells share the same signaling mechanisms.

It was shown that the Rho family of small G-protein play an essential role in cancer metastasis, (96). It was demonstrated that overexpression of Rho proteins increased the invasive potential of melanoma cells and atorvastatin caused disruption of stress fibers as a result of Rho inhibition therefore decreasing the metastatic potential in melanoma cells. Similarly, as a result of Rho inhibition, statins were shown to reduce the invasive potential of breast carcinoma cells (97).

In our experimental data we obtain that atorvastatin administration drastically inhibits the expansion of mammosphere- derived cells. This result could be driven by inhibition RhoA pathway, indeed recently work reported that statins suppress self-renewal and differentiation (95) in mouse embryonic stem cells by RhoA-mediated inhibition (98).

Our results suggest that atorvastatin is able to modulate different Her2 dependent signaling pathways. This feature has allowed that in other contexts to consider a statin drug very versatile, but in our case may paradoxically dissipate the expected beneficial effect of HMGCoAR inhibitors in terms of cell proliferation.

It is therefore important to identify the signaling pathway that controls proliferation, and atorvastatin can be used as an adjuvant to specific cancer therapy.

In particular, our mouse model of spontaneous carcinogenesis allowed us to understand that to inhibit tumor onset is needed to change the mammary microenvironment, in fact transformed cells are subjected to various local stimuli such as growth factors, cytokines and stromal cell, that may interact with anti-tumor therapy.

The data obtained with atorvastatin on CIC, leave an open door on the use of this drug, not only as an adijuvant in cancer therapy but as preventive drug of metastases generation.

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