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Several oncogenes, growth factors, hormones and hypoxia have been shown to up-regulate VEGF, an essential angiogenic factor for the progression of breast carcinomas. The angiogenic factor CYR61, a ligand for the $\alpha_v\beta_3$ integrin, is differentially up-regulated in invasive and metastatic breast cancer cells overexpressing the epidermal growth factor-like growth factor Heregulin (HRG). HRG can regulate breast cancer neovascularization through its ability to activate the expression and secretion of VEGF. Although CYR61 is thought to be an angiogenic ligand for $\alpha_v\beta_3$ integrin in endothelial cells, little is know about the regulatory role of CYR61 on the secretion of VEGF in the epithelial compartment of breast carcinoma. We speculated that CYR61 may promote VEGF-dependent breast cancer angiogenesis in an autocrine fashion, and we examined whether HRG-induced over-secretion of VEGF in breast cancer cells associated with an increased CYR61-regulated $\alpha_v\beta_3$ integrin signaling. First, constitutive VEGF secretion positively correlated with HRG overexpression, but not with Her-2/*neu* (*erb*B-2) oncogene status, in a panel of human breast cancer cell lines. Second, we evaluated the levels of VEGF secretion in MDA-231 cells, a natural breast cancer model overexpressing HRG, in which HRG expression was diminished using an HRG antisense (AS) cDNA. Secretion of VEGF was significantly diminished in the unselected MDA-MB-231/AS-POOL population (up to 30% reduction), and decreased by about 55% in MDA-231/AS-31 transfectants, an AS-HRG clone expressing low to undetectable levels of HRG. Since blockade of HRG expression in MDA-231 cells concomitantly down-regulated the expression of $\alpha_v\beta_3$ and CYR61, we next used CYR61-, HRG-, and $\alpha_v\beta_3$-negative MCF-7 cells to study the effects of CYR61 overexpression in VEGF secretion. MCF-7 cells engineered to overexpress CYR61 cells exhibited higher levels of $\alpha_v\beta_3$ and they became insensitive to the cytotoxic Taxol, an anti-angiogenic agent. CYR61-overexpressing MCF-7 cells concomitantly secreted higher levels of VEGF, while the anti-sense down-regulation of CYR61 expression reduced VEGF secretion in MCF-7 engineered to overexpress HRG. Moreover, functional blocking of $\alpha_v\beta_3$ signaling using small peptidomimetic $\alpha_v\beta_3$ integrin antagonists significantly reduced VEGF secretion in HRG- and CYR61-overexpressing breast cancer cells, while sensitizing them to Taxol-induced apoptotic cell death. Therefore, a previously unrecognized CYR61-$\alpha_v\beta_3$ signaling synergistically cooperates with HRG-induced transactivation of Her-2/*neu* in order to maintain high levels of VEGF secretion and promote chemoresistance in metastatic breast cancer cells. Current and future antagonists directed against $\alpha_v\beta_3$, or more specific anti-HRG and anti-CYR61 strategies, may have the potential to suppress HRG- and CYR61-promoted metastatic phenotype in breast cancer disease.

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INTRODUCTION

Accumulating evidence supports an association between angiogenesis and the processes of tumor invasion and metastasis. In this way, several angiogenic factors and their receptors have been identified as important mediators of angiogenesis (1). We as well others have accumulated evidence suggesting that **HEREGULIN** (HRG), a growth factor involved in the progression of breast cancer to a more malignant phenotype (2-4), also is a potent stimulator of *in vitro* growth of a special blood vessel cells found in the umbilical cord, called HUVEC (Human Umbilical Vein Endothelial Cells). Hence, HRG pathway can enhance tumor neovascularization and this up-regulation of angiogenesis may contribute to a more aggressive disease. Significantly, we have also demonstrated that the growth factor-inducible immediate-early gene **CCN1** (Cysteine-rich angiogenic protein 61; **CYR61**) is a down-stream effector of HRG-induced breast cancer chemomigration and metastasis, probably through interactions with the **$\alpha_v\beta_3$ integrin receptor** (5). CCN1 stimulates chemotaxis in endothelial cells and induce neovascularization *in vivo* (6). Moreover, we have shown that CCN1 overexpression in tumor cells enhances tumorigenicity by increasing tumor size and vascularization (7). In this regard, we have determined previously that CCN1 gene expression is elevated in highly invasive and metastatic human breast cancer cells and tumor biopsies (5). Accordingly, CCN1 overexpression is correlated with more advance stage of malignancy in patients samples (7). Taken together, these findings prompted us to hypothesize that HRG (directly or indirectly through CCN1) is an important regulator of the vascular compartment in breast cancer with stimulating effects on tumor neovascularization which, in turn, promotes progression and dissemination of breast carcinoma.

We recently showed that in ovariectomized nude mice brast carcinoma cells secreting HRG promoted more vascularized tumors (9). We demonstrated that one of the mechanisms by which HRG achieved this aggressive phenotype was mediated *via* an increase in the expression of Vascular Endothelial Growth Factor (VEGF), a key tumor angiogenic factor. In MCF-7/HRG-derived tumors, a great increase in VEGF expression was observed by inmunohistochemistry staining with anti-VEGF antibody. These results were further confirmed by an ELISA assay, demonstrating a 3- to 8-fold increase in VEGF expression was observed in the conditioned media from HRG-transfected cells. Of interest, in our experiments there was a positive correlation between the increase in the ability of the HRG transfectants to secrete VEGF and the levels of HRG expression. Consistent with this finding, HRG has been shown to selectively up-regulate VEGF secretion in both cancer and HUVEC cells and to stimulate *in vivo* angiogenesis (10). Nevertheless, some of the effects that were observed *in vivo* were probably mediated indirectly *via* the up-regulation of other genes in an autocrine/paracrine manner. For example, the expression of CCN1 was significantly up-regulated in the MCF-7/HRG-derived tumors. Accordingly, we found that CCN1-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of VEGF (7).

An important issue that arises from the contribution of VEGF to breast cancer neovascularization is an understanding of the mechanism(s) that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in breast cancer cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (11), but other factors are likely to be involved. Of note, our finding that the **$\alpha_v\beta_3$** integrin can promote the survival of breast carcinoma cells in stress conditions such as chemotherapy treatment is intriguing (12), and raised the novel possibility that a specific integrin $\alpha_v\beta_3$, which has been implicated in breast cancer progression (13), could play an active role regulating VEGF secretion in HRG-overexpressing breast cancer cells. Moreover, virtually every conventional cytotoxic anti-cancer drug has been "accidentally" discovered to have anti-angiogenic effects in various *in vivo* models (14,15), whereas it has been suggested that exploiting chemotherapeutic drugs as anti-angiogenics is likely to be compromised by the high-concentrations of pro-angiogenic/survival/growth

factors present in the tumor microenvironment (16). Because the pro-angiogenic abilities of HRG and CYR61, we also envisioned that HRG could act directly, or indirectly through CYR61, as a survival factor for both tumor and endothelial cells, thus modifying the efficacy of chemotherapy in breast carcinomas.

BODY

The main goal in the proposed study was to determine the effect of HRG on the secretion of angiogenic factors and elucidate the contribution of HRG to the angiogenic potential of breast carcinomas. Since VEGF appears to be an essential angiogenic factor for the progression of many solid tumors, including breast carcinomas, we examined the role of HRG on the regulation of the VEGF secretory isoform, $VEGF_{165}$, in relation to the expression level of either CCN1 (CYR61) or Her-2/*neu* oncogene in human breast cancer cells.

Accumulating evidence supports an association between angiogenesis and the processes of breast cancer invasion and metastasis (17-20). In this regard, several angiogenic factors and their receptors have been identified as important mediators of angiogenesis (21-24). We as well others have accumulated evidence suggesting that Heregulin (HRG), an activator of *erb*B-2/-3/-4 receptor signaling pathways closely involved in the progression of breast cancer to a more malignant phenotype (2, 4, 9, 25, 26), is also a potent stimulator of breast cancer angiogenesis (9, 10, 27). Accordingly, we recently showed that in ovariectomized nude mice, breast cancer cells secreting HRG promoted more vascularized tumors (9). We demonstrated that one of the mechanisms by which HRG achieved this aggressive breast cancer phenotype was mediated *via* an increase in the expression of Vascular Endothelial Growth Factor (VEGF), an angiogenic factor of reference (28-30). In HRG-derived tumors, a great increase in VEGF expression was observed by inmunohistochemistry staining with an anti-VEGF antibody. Of interest, in our experiments there was a positive correlation between the increase in the ability of the HRG transfectants to secrete VEGF and the levels of HRG expression. Consistent with these findings, HRG has been shown to selectively up-regulate VEGF secretion in both cancer and endothelial (HUVEC) cells and to stimulate *in vivo* angiogenesis (10). Nevertheless, some of the effects that were observed *in vivo* were probably mediated indirectly *via* the up-regulation of other genes in an autocrine/paracrine manner.

We recently isolated and identified CYR61 (CCN1; the human homologue of a mouse immediate early response gene, *Cyr61*), an angiogenic regulator that is differentially expressed in invasive and metastatic human breast cancer cells (5). CYR61, a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (31), belongs to the Cysteine rich 61/Connective tissue growth factor/Nephroblastoma overexpressed (CCN) gene family of angiogenic and growth regulators, which consists of CCN1 (CYR61), CCN2 (CTFG), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (6-8, 12, 38). All CCN proteins, including CYR61, have been shown to mediate functions as diverse as cell proliferation, migration, adhesion, cell survival, differentiation, and extracellular matrix formation. Remarkably, CYR61 also regulates more complex processes, such as angiogenesis and tumorigenesis (6-8, 12, 38, 39). In the breast cancer scenario, we have previously shown that CYR61 is a down-stream effector of HRG-dependent breast cancer aggressiveness (5, 7, 12). Moreover, we established that forced expression of CYR61, in the absence of HRG and *erb*B-2 oncogene overexpression, is sufficient to promote acquisition of hormone independence and anti-estrogen resistance in human breast cancer cells (7). Indeed, CYR61 enhances a metastatic phenotype by promoting cell proliferation in soft agar, cell migration and invasion, and Matrigel outgrowth of breast cancer cells (5). Accordingly, CYR61 overexpression is correlated with more advance stage of malignancy in patient samples (8, 12, 40). Although these results, taken together, strongly indicate that CYR61 may play a key role in the process of breast cancer development and might serve as valuable tool for monitoring the tumor

status, the ultimate mechanisms by which CYR61 promotes an aggressive breast cancer phenotype are still largely unknown.

We have recently shown that CYR61 overexpression enhances tumorigenicity by increasing tumor size and vascularization of human breast cancer xenografts (7). CYR61-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of VEGF (7). These findings prompted us to hypothesize that CYR61 (dependently or independently of HRG) is an important regulator of the vascular compartment in breast cancer with stimulating effects on tumor neovascularization which, in turn, promotes progression and dissemination of breast carcinoma. Considering that CYR61 is an angiogenic ligand for $\alpha_v\beta_3$ integrin receptor in endothelial cells (41-43), it would be reasonable to suggest that CYR61 is mediating breast cancer angiogenesis in a paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor. However, little is know about the regulatory role of CYR61 on the secretion of VEGF in the epithelial compartment of breast carcinoma. We herein speculated that CYR61 may promote VEGF-dependent breast cancer angiogenesis in an autocrine fashion, and we examined whether HRG-induced over-secretion of VEGF in breast cancer cells associates with an increased CYR61-$\alpha_v\beta_3$ integrin signaling in the epithelial compartment of breast carcinomas.

In this final report, we demonstrate that HRG-stimulated secretion of $VEGF_{165}$ in human breast cancer cells requires an autocrine action of HRG on Her-2/*neu*-dependent signaling. In this regard, we generated a deletion mutant of HRG (HRG-M4) lacking the N-terminus sequence and the cytoplasmic-transmembrane region of HRG protein, which did not stimulate either Her-2/*neu* phosphorylation or $VEGF_{165}$ secretion. Interestingly, we provide the first evidence that CYR61 stimulates $VEGF_{165}$ secretion, and concomitantly breast cancer cell resistance to Taxol-induced cell damage, independently of HRG overexpression and/or Her-2/*neu* activation. Moreover, we reveal that CYR61 synergistically enhances HRG-stimulated secretion of VEGF *via* activation of MAPK and phosphatidylinositol 3'-kinase (PI-3'K)/protein kinase B (AKT) signaling pathways. In addition, we show that the involvement of CYR61 in $VEGF_{165}$ secretion can be attributed, at least in part, to the ability of CYR61 to up-regulate the expression of its own $\alpha_v\beta_3$ integrin receptor in human breast epithelial cells.

1. Constitutive VEGF secretion correlates with HRG expression in human breast cancer cell lines. Figure 1a shows the basal level of the VEGF secretory isoform, $VEGF_{165}$, in human breast cancer cell lines naturally overexpressing either the Her-2/*neu* oncogene (SK-Br3 and BT-474) or the Her-2/*neu* transactivator HRG (MDA-MB-231, Hs578T) when compared to $VEGF_{165}$ secretion levels in MCF-7 breast cancer cells, which express physiological levels of Her-2/*neu* and HRG. HRG-overexpressing MDA-MB-231 and Hs578T breast cancer cell lines demonstrated $VEGF_{165}$ secretion levels (20.2 ± 3 and 28.8 ± 0.5 pg VEGF/μg protein, respectively) significantly beyond the up-regulation mediated by overexpression of Her-2/*neu* oncogene in SK-Br3 and BT-474 breast cancer cell lines (10.4 ± 1.8 and 11.9 ± 0.1 pg VEGF/μg protein, respectively), when compared with low Her-2/*neu*- and HRG-expressing MCF-7 cells (4.2 ± 0.2 pg VEGF/μg protein).

2. Forced expression of Heregulin up-regulates VEGF secretion in breast cancer cells. To further evaluate whether HRG overexpression, independently of Her-2/*neu* overexpression, up-regulates VEGF secretion, we looked at MCF-7 cells that express low levels of Her-2/*neu*, and the same cells engineered to overexpress HRG (MCF-7/T clones; Figure 1*b*). The basal level of VEGF secretion was likewise significantly increased in MCF-7/HRG transfectants (up to 15.7 ± 1.3 pg VEGF/μg protein in the MCF-7/T7 clone). This observation was not a clonal selection effect since a significant induction of $VEGF_{165}$ secretion was found in MCF-7 cells stably transduced with a pBABE retroviral vector containing the identical HRG

cDNA (data not shown). These results, altogether, strongly suggest that the status of HRG expression, but not the amplification and/or overexpression of Her-2/*neu* oncogene, positively correlates with VEGF secretion levels in human breast cancer cells.

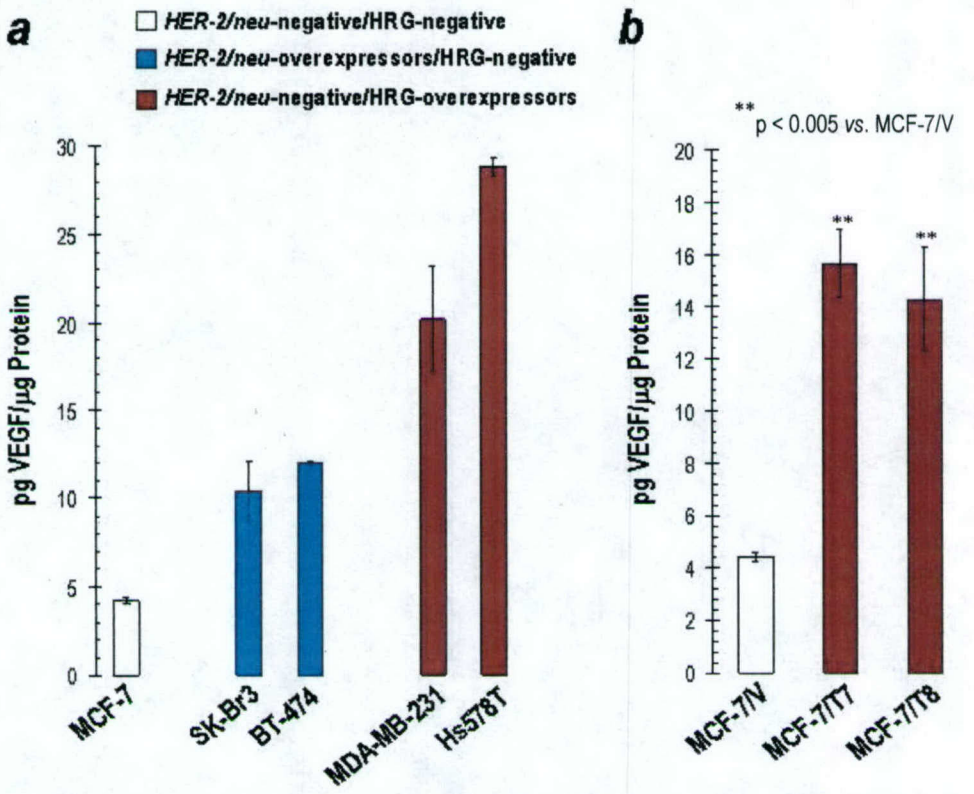
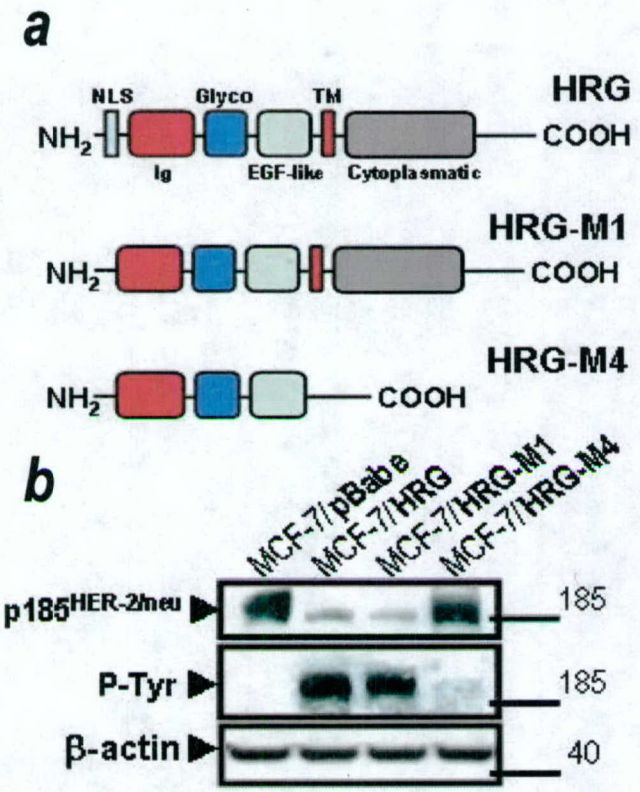


Figure 1. ***a.*** Human breast cancer cell lines with constitutive overexpression of either Her-2/*neu* (erbB-2) *erb*B-2 (SK-Br3 and BT-474) or HRG (MDA-MB-231 and Hs578T) were overnight serum starved, and then cultured in 0.1% FBS-IMEM for 48 h. The level of $VEGF_{165}$ in the supernatant was determined by a $VEGF_{165}$-specific ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in Her-2/*neu*- and HRG-negative MCF-7 cells. ***b.*** MCF-7 cells engineered to overexpress HRG (MCF-7/T7 and MCF-7/T8 clones) were overnight serum starved, and then cultured in 0.1% FBS-IMEM for 48 h. The level of $VEGF_{165}$ in the supernatant was determined by a $VEGF_{165}$-specific ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in matched control MCF-7/*V* cells.

3. HRG-stimulated secretion of $VEGF_{165}$ requires an autocrine action of HRG on Her-2/*neu*-dependent signaling. HRG-induced responses are mainly mediated by the *erb*B family of tyrosine kinase receptors (*Her-2/-3/-4*). MCF-7/T clones and MCF-7/HRG cells produce high levels of HRG and thus have constitutively activated *HER-2/neu* receptors in spite of their low *HER-2/neu* receptors expression (Figure 2). To investigate whether up-regulation of $VEGF_{165}$ secretion plays a role in HRG-promoted aggressive phenotype of breast cancer cells, we evaluated $VEGF_{165}$ levels in MCF-7 cells engineered to express a deletion mutant of HRG (HRG-4) incapable of promoting tumorigenicity (44). HRG-M4 is a structural mutant of HRG β-2 that lacks N-terminus sequences (a putative nuclear localization signal -NLS-) and the cytoplasmic domain of the protein (Figure 2*a*). We previously demonstrated that HRG-4 protein, although stably expressed in MCF-7 cells, is sequestered into a cellular compartment and is not secreted into the culture media, thus preventing its autocrine action and Her-2/*neu* autophosphorylation. In addition, MCF-7/HRG-M4 cells did not become more aggressive or estrogen-independent, which was opposed to the phenotype arising from the full-length HRG protein (44). Here, MCF-7 cells were transduced with the



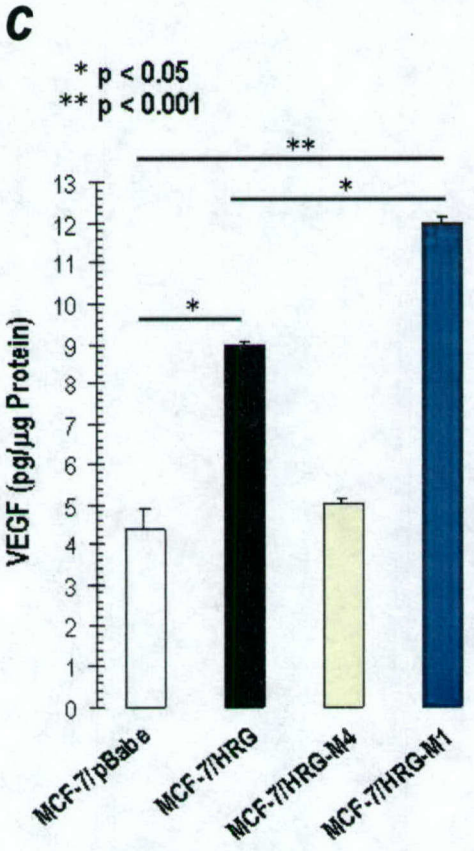


Figure 2. Relationship between HRG cellular localization and $VEGF_{165}$ secretion in breast cancer cells.

deletion mutant of HRG (MCF-7/HRG-M4 cells) or with the empty retroviral vector (MCF-7/pBabe) to circumvent the possibility of clone variations, and a stable MCF-7/HRG-M4 cell line was expanded after selection in puromycin containing media. As expected, Western blotting analysis of MCF-7/HRG-M4 cells demonstrated neither down-regulation of $p185^{HER\text{-}2/neu}$ receptor nor increase in $p185^{HER\text{-}2/neu}$ tyrosine phosphorylation when compared with the matched control cells (Figure 2*b*). Interestingly, the transduction of MCF-7 cells with the HRG deletion mutant M4 did not cause any increase in $VEGF_{165}$ secretion when compared to the empty-vector infected MCF-7 cells (Figure 2*c*). This result indicates that the deletion of the NLS sequence and the cytoplasmic domain of the full-length HRG protein abolish its ability to up-regulate $VEGF_{165}$ secretion in MCF-7 breast cancer cells. Moreover, HRG-induced over-secretion of $VEGF_{165}$ is likely dependent from activation of the Her-2/*neu*-dependent signaling.

4. HRG-stimulated secretion of $VEGF_{165}$ does not require a nuclear localization of HRG. HRG secretion to the extracellular media activates the *erb*B receptors. Interestingly, HRG also localizes in the nuclei as previously observed in MCF-7 breast cancer cells transfected with the HRG cDNA (44). It is not clear, however, which functions can be attributed to the nuclear HRG and which functions, if any, can be independent of Her-2/*neu* receptor activation. Therefore, we investigated whether secretion of HRG followed by activation of Her-2/*neu* receptor are *necessary* and/or *sufficient* molecular events in the HRG-promoted secretion of $VEGF_{165}$. Our previous studies, using GFP-tagged HRG demonstrated a clear nuclear localization of the extracellular domain of the protein (44). Interestingly, this localization was independent of the localization of the *erb*B receptors, which have been previously identified in the nucleus of mammary epithelial cells, as assessed by co-localization experiments using confocal microscopy (data not shown). Therefore, we concluded that HRG must have a NLS at the NH_2-terminus. Likewise, we identified a novel NLS in the extracellular domain of the HRG-protein between the fourth and the sixteenth amino acids, which does not fully resemble any of the known nuclear localization sequences, but has close homology to the NLS

that is found in the p53 protein. In order to confirm its functionality, we deleted the first 33 amino acids of the HRG sequence, containing the putative NLS, and replaced it with GFP (NLS-) HRG. The HRG-negative, mammary epithelial breast cancer cell line MCF-7, was transfected with the (NLS-) HRG or full–length HRG-GFP fusion expression plasmids, and the localization of the fusion proteins was visualized by confocal microscopy. We observed a perinuclear localization of the HRG protein lacking the NLS sequence, which was markedly different from the clearly nuclear localization of the full-length HRG protein (data not shown). These results confirm that HRG contains a functional NLS, which is essential for the translocation of the growth factor to the nucleus in MCF-7 cells. Based on these studies we set to identify if the nuclear localization of HRG was required for the up-regulation of $VEGF_{165}$ secretion. We found that the expression of the HRG-M1 increased the expression of $VEGF_{165}$ to even higher levels then the full-length HRG (Figure 2*c*). We investigated whether the phenotypic changes that are mediated by deletion of the NLS of HRG are mediated through changes in the levels of Her-2/*neu* phosphorylation. Conversely to HRG-M4, as HRG-M1 protein is secreted, MCF-7/HRG-M1 cells did behave similarly to the MCF-7 cells infected with the wild-type HRG, and demonstrated down-regulation of Her-2/*neu* protein expression and constitutive activation of $p185^{HER\text{-}2/neu}$ (Figure 3*b*). These results indicate that the nuclear NH_2-terminus of HRG acts as a suppressor rather than an activator of $VEGF_{165}$ secretion in human breast cancer cells.

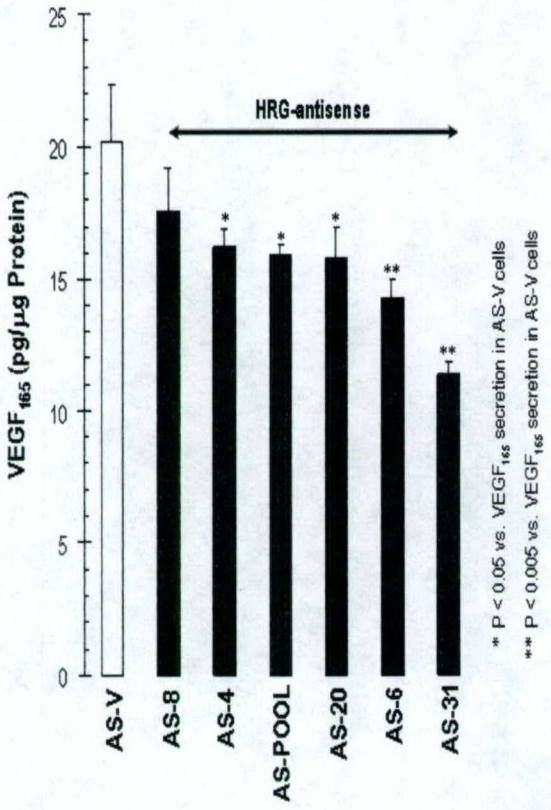


Figure 3. HRG-overexpressing MDA-MB-231 and antisense-HRG derivatives were overnight serum starved, and then cultured in 0.1% FBS-IMEM for 48 h. The level of $VEGF_{165}$ in the supernatant was determined by a VEGF-specific ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in matched control AS-V cells.

5. Blockade of HRG expression concomitantly down-regulates VEGF, $\alpha_v\beta_3$ and CYR61 in MDA-MB-231 breast cancer cells. Considering that HRG overexpression concomitantly up-regulates the expression of pro-angiogenic factor CYR61 (5), we employed four different approaches to evaluate the role of CYR61 in HRG-induced over-secretion of VEGF. In the first scenario, we used HRG-overexpresssing MDA-MB-231, which express low to undetectable levels of Her-2/*neu*. Thus, the receptor signaling events that lead to over-secretion of VEGF in this cellular system are not dependent upon Her-2/*neu* overexpression. We evaluated the levels of VEGF secretion in MDA-MB-231 cells in which HRG overexpression was diminished by transfection with the HRG cDNA oriented from 3' to 5' end, that is, in an antisense direction (26). Conditioned media from an unselected population of HRG-AS clones (AS-POOL), and several representative clones (AS-4, AS-6,

AS-8, AS-20 and AS-31) were analyzed for VEGF secretion levels and compared to those found in empty vector-transfected MDA-MB-231 (MDA-MB-231/AS-V) cells (Figure 3). VEGF secretion was significantly reduced in the AS-POOL population, and dramatically decreased by about 55% in MDA-MB-231/AS-31 transfectants (from 20.2 ± 2 in AS-V to 11.4 ± 0.5 pg VEGF/μg protein in AS-31) which express low to undetectable levels of HRG (12).

To understand the mechanism by which blockade of HRG expression down-regulated VEGF secretion, we examined whether $\alpha_v\beta_3$ and/or CYR61 expression were altered in HRG-AS cells. We recently demonstrated that blocking HRG expression using an HRG antisense cDNA significantly down-regulates $\alpha_v\beta_3$ overexpression in MDA-MB-231 breast cancer cells (45). Moreover, when we examined whether the decreased level of HRG expression in the HRG-AS clones also correlated with changes in CYR61 expression, a significant reduction in CYR61 expression was found in whole lysates and cell supernatants from AS-POOL, AS-6 and AS-31 cells (45). These findings demonstrate that the decreased levels of CYR61 and $\alpha_v\beta_3$ expression in the HRG-AS MDA-MB-231 transfectants correlate with their decreased ability to secrete VEGF.

6. Forced expression of CYR61 up-regulates VEGF secretion in breast cancer cells. On the basis of these results, we envisioned that high levels of CYR61 and of its $\alpha_v\beta_3$ integrin receptor would be a prerequisite for the high levels of VEGF secretion found in HRG-overexpressing breast cancer cells. To assess this hypothesis, we evaluated whether forced expression of CYR61 did modify VEGF basal levels in MCF-7 cells, which express very low to undetectable levels of CYR61, HRG, and Her-2/*neu* (Figure 4). Likewise, MCF-7 cells engineered to overexpress CYR61 demonstrated $VEGF_{165}$ secretion levels (up to 10.2 ± 0.1 pg VEGF/μg protein in MCF-7/C2-2 clone) significantly higher than those found MCF-7/pBABE control cells (4.2 ± 0.8 pg VEGF/μg protein). We also assessed the expression and the activation status of Her-2/*neu* in the control empty-vector and the MCF-7 clones stably expressing CYR61. As expected, no significant changes in either the expression or tyrosine phosphorylation of Her-2/*neu* was detected in MCF-7/CYR61 clones (data not shown).

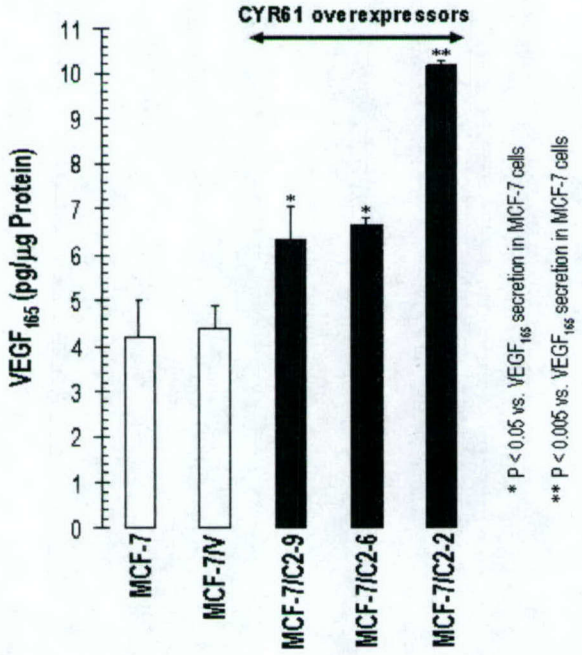


Figure 4. MCF-7 cells engineered to overexpress CYR61 (MCF-7/C2-6, MCF-7/C2-9, and MCF-7/C2-2 clones) were overnight serum starved, and then cultured in 0.1% FBS-IMEM for 48 h. The level of $VEGF_{165}$ in the supernatant was determined by the ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in MCF-7cells.

7. CYR61 overexpression up-regulates $\alpha_v\beta_3$ integrin in breast cancer cells. We envisioned that high levels of CYR61 would be a prerequisite for the high levels of $\alpha_v\beta_3$ integrin found in HRG-overexpressing breast cancer cells. To assess this hypothesis we took advantage of a recent cellular model of breast cancer CYR61 overexpression developed in our laboratory, in which non-tumorigenic and estrogen-dependent MCF-7 cells, which express very low to undetectable levels of CYR61, HRG, and *erb*B-2, were stably transfected with the full-length cDNA of CYR61 (7). To determine if overexpression of CYR61 in breast cancer cells altered the levels of its integrin receptor $\alpha_v\beta_3$ in the absence of HRG overexpression, the cell surface expression of $\alpha_v\beta_3$ in two representative CYR61 derivatives (MCF-7/C2-6 and MCF-7/C2-9 clones) was assessed by flow cytometry of cells stained with the anti-$\alpha_v\beta_3$ monoclonal antibody LM609. As control, the cells were stained with the appropriate antibody isotype. In MCF-7 breast cancer cells transfected with an empty vector (MCF-7/V), which expresses very low levels of CYR61, $\alpha_v\beta_3$ integrin was almost not detectable by flow cytometry (Figure 5*a*). Interestingly, an equivalent flow cytometric analysis demonstrated a very significant increase of $\alpha_v\beta_3$ levels in CYR61-transfected MCF-7/C2-6 and MCF-7/C2-9 clones (Figure 5*a*).

To confirm the results obtained by flow cytometry we next analyzed the impact of CYR61 overexpression in the sub-cellular localization of $\alpha_v\beta_3$. To address this question, CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones and matched control MCF-7/V cells were permeabilized with Triton X-100 for the intracellular delivery of an anti-$\alpha_v\beta_3$ monoclonal antibody, and then the cellular pattern of $\alpha_v\beta_3$ was assessed by immunofluorescence microscopy. The membrane staining of $\alpha_v\beta_3$ was almost undetectable in MCF-7/V control cells (Figure 5*b*). Conversely, CYR61-overexpressing MCF-7/C2-6 and MCF/C2-9 transfectants showed a prominent cell-surface staining of $\alpha_v\beta_3$ (Figure 5*b*). In fact, $\alpha_v\beta_3$ integrin in CYR61-overexpressing MCF-7 cells was to some extent distributed throughout the cytoplasm. Although direct quantitative interpretation of immunofluorescence is not possible, comparison of the intensity of $\alpha_v\beta_3$ dots observed with the $\alpha_v\beta_3$-specific antibody revealed an obvious and highly reproducible difference between CYR61 transfectants and control cells. Up-regulation of $\alpha_v\beta_3$ by CYR61 overexpression was also evident when $\alpha_v\beta_3$ expression levels were monitored by immunoblotting (Figure 5*c*, *top*). When the expression levels of $\alpha_v\beta_3$ were quantitatively evaluated using an expression index (Geo Mean Fluoresence *x* % of $\alpha_v\beta_3$-positive cells –M1; Figure 5*a*), this approach clearly demonstrated that CYR61 overexpression dramatically increased (> 200 times) the basal level of $\alpha_v\beta_3$ expression in MCF-7 breast cancer cells (Figure 5*c*, *bottom*). CYR61 was highly expressed in the CYR61 transfectants as determined by Western blotting analysis using conditioned media concentrated from the C2-6 and C2-9 clones or a vector control clone (Figure 5*c*, *top*). Indeed, the expression level of CYR61 in MCF-7/C2-6 and MCF-7/C2-9 clones was comparable to that in MDA-MB-231 cells (an aggressive breast cancer cell line naturally overexpressing HRG and CYR61), as compared with the wild-type MCF-7 or MCF-7/V cells, in which CYR61 was nearly undetectable. This is the first indication showing that up-regulation of $\alpha_v\beta_3$ integrin expression in human breast epithelial cells can be achieved solely by CYR61 overexpression, irrespective of HRG status. Importantly, the basal level of $\alpha_v\beta_3$ expression was significantly higher in MDA-MB-231 cells as compared with MCF-7 cells engineered to overexpress CYR61. Therefore, in breast cancer cells naturally overexpressing both HRG and CYR61 (*i.e.* MDA-MB-231 cells), is likely that HRG further enhances CYR61-stimulated $\alpha_v\beta_3$ expression in a synergistic manner. We are currently investigating the ultimate mechanism responsible for the exacerbated levels of $\alpha_v\beta_3$ in MDA-MB-231 breast cancer cells.

Considering that CYR61 overexpression dramatically up-regulates the expression of its own receptor $\alpha_v\beta_3$ in MCF-7 cells (46), these *in vitro* findings, altogether, suggest that CYR61 overexpression, in an $\alpha_v\beta_3$-related manner, is *sufficient* to up-regulate $VEGF_{165}$ secretion in the absence of HRG-induced transactivation of Her-2/*neu* tyrosine kinase receptor.

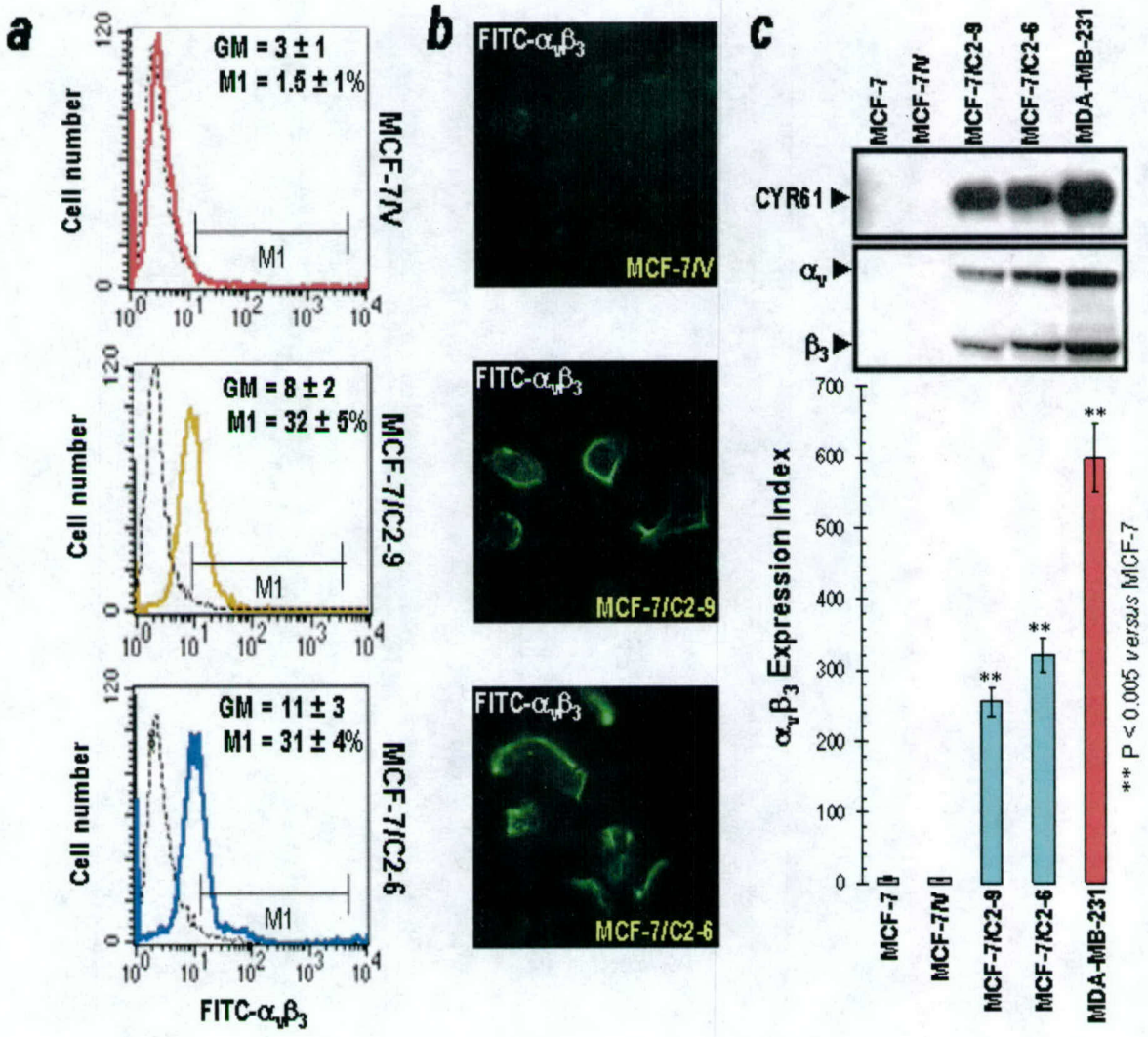


Figure 5. CYR61 overexpression up-regulates $\alpha_v\beta_3$ integrin expression in MCF-7 cells. ***a.*** Flow cytometric quantification of $\alpha_v\beta_3$ integrin expression in CYR61-derivatives MCF-7/C2-6 and C2-9 clones, and matched control MCF-7/V cells. The specific surface expression of $\alpha_v\beta_3$ in CYR61-overexpressing MCF-7/C2-6 and C2-9 clones, and matched control MCF-7/V cells was determined by flow cytometry by measuring the binding of a mouse anti-$\alpha_v\beta_3$ antibody (clone LM609; Chemicon). The data presented summarize the mean of the Geo Mean (GM) fluorescence parameter (± S.D.) from three independent experiments. ***b.*** *In situ* immunofluorescent staining of $\alpha_v\beta_3$ integrin in CYR61-derivatives MCF-7/C2-6 and C2-9 clones, and matched control MCF-7/V cells. ***c.*** Correlation between the expression of CYR61 and $\alpha_v\beta_3$ in breast cancer cells. *Top.* Sub-confluent wild-type MCF-7, MCF-7/V, MCF-7/C2-9, MCF-7/C2-6 and MDA-MB-231 breast cancer cells were maintained in serum-free media for 48 h in 100-mm plates. The conditioned media were collected, concentrated 20*x*, resolved by 10% Tris-glycine SDS-PAGE, and assayed for CYR61 expression using a rabbit anti-CYR61 polyclonal antibody (ab2026; 1:2,000 dilution). 50 μg weight of whole cell lysates from the same experimental 100-mm plate were resolved by 10% Tris-glycine SDS-PAGE, transferred to nitrocellulose membranes, and then probed with a mouse monoclonal anti-$\alpha_v\beta_3$ antibody. *Bottom.* A $\alpha_v\beta_3$ expression index was calculated using the formula GM (Geo Mean Fluoresence) *x* % of $\alpha_v\beta_3$-positive cells. Data presented summarize the mean (*columns*) ± S.D. (*bars*) of three independent experiments.

8. Blockade of CYR61 expression down-regulates VEGF secretion in breast cancer cell engineered to overexpress Heregulin. In the light of these observations we hypothesized that a CYR61-$\alpha_v\beta_3$

autocrine loop may be an angiogenic-promoting signaling that acts independently of HRG overexpression. To evaluate this hypothesis, we characterized VEGF secretion levels following blockade of CYR61 expression in MCF-7 cells engineered to overexpress HRG, which show a constitutive hyperactivation of Her-2/*neu*. Conditioned media from CYR61 AS-2, AS-4, AS-6, and AS-7 were collected and the secretion levels of $VEGF_{165}$ were determined as previously described. Figure 6 demonstrates the effect of antisense down-regulation of CYR61 expression on the level of $VEGF_{165}$ in the HRG-overexpressing MCF-7/T7 clone. Interestingly, blockade of CYR61 expression in HRG-overexpressing MCF-7/T7 cells led to dramatic decreases in HRG-induced $VEGF_{165}$ stimulation. However, the blockade of CYR61 expression in MCF-7 cells engineered to overexpress HRG was not able to reduce $VEGF_{165}$ secretion to the basal level observed in the wild type or MCF-7/V control cells. Importantly, the levels of $p185^{Her\text{-}2/neu}$ receptor expression and its phosphorylation (*e.g.*, activation) status were unchanged in antisense-CYR61 transfectants when compared with those found in T7 and T7/AC-V controls (46).

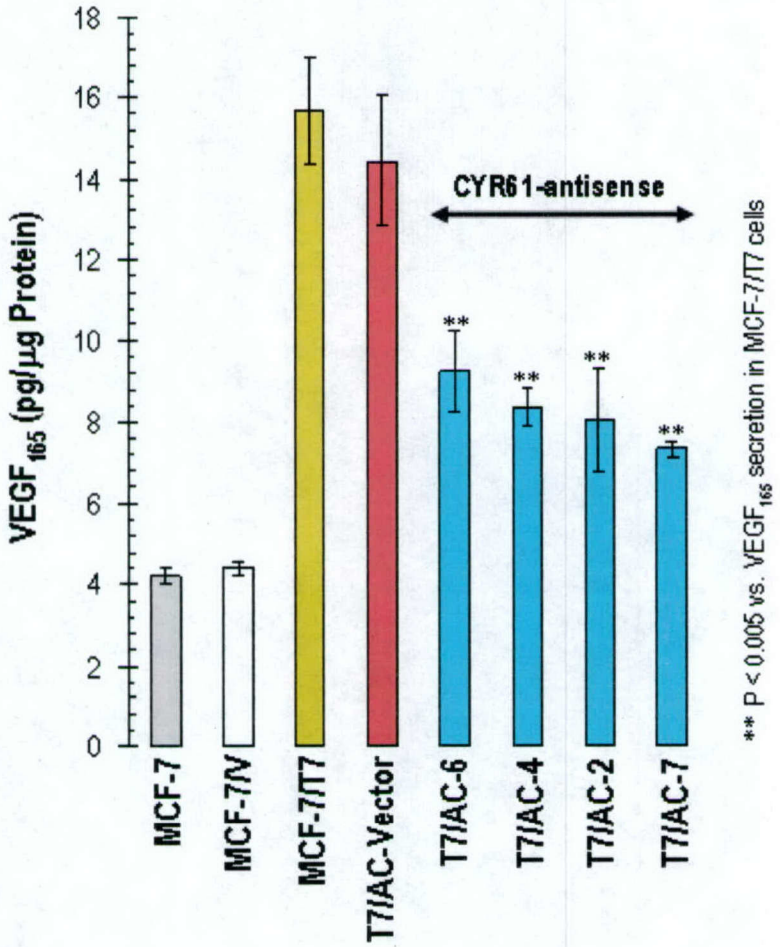


Figure 6. HRG-overexpressing MCF-7/T7 cells and antisense-CYR61 derivatives were overnight serum starved, and then cultured in 0.1% FBS-IMEM for 48 h. The level of $VEGF_{165}$ in the supernatant was determined using a $VEFG_{165}$-specific ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in MCF-7/T7 cells.

9. Functional blockade of $\alpha_v\beta_3$ down-regulates VEGF secretion in Heregulin- and CYR61-overexpressing breast cancer cells. To further demonstrate the active involvement of CYR61 and $\alpha_v\beta_3$ in the maintenance of high levels of VEGF in breast cancer cells, we finally assessed VEGF secretion levels in CYR61- and HRG-overexpressing MCF-7 cells following exposure to a novel group of small peptidomimetic antagonists of $\alpha_v\beta_3$ (SC56631, SC68448, S-247, S-197, and S-205; Oncology Pharmacology, Discovery Research, Pharmacia Corporation, St. Louis, MO). CYR61-overexpressing MCF-7/C2-6 cells incubated in the presence of S-247, the $\alpha_v\beta_3$ antagonist with the highest affinity and specificity for $\alpha_v\beta_3$, significantly decreased their levels of $VEGF_{165}$ secretion (Figure 7, *left panel*). Similarly, a significant inhibitory effect on $VEGF_{165}$ secretion was induced by S-247 in MCF-7 cells engineered to overexpress HRG (T7 clone; Figure 7, *right panel*). These results, altogether, demonstrate that CYR61 and $\alpha_v\beta_3$ are *necessary* for the optimal stimulation of $VEGF_{165}$ secretion in HRG-overexpressing breast cancer cells. Therefore, it is reasonable to suggest that a CYR61-$\alpha_v\beta_3$ autocrine loop synergistically cooperates with HRG-induced transactivation of Her-2/*neu*-driven signaling in order to maintain very high levels of VEGF secretion in breast cancer cells.

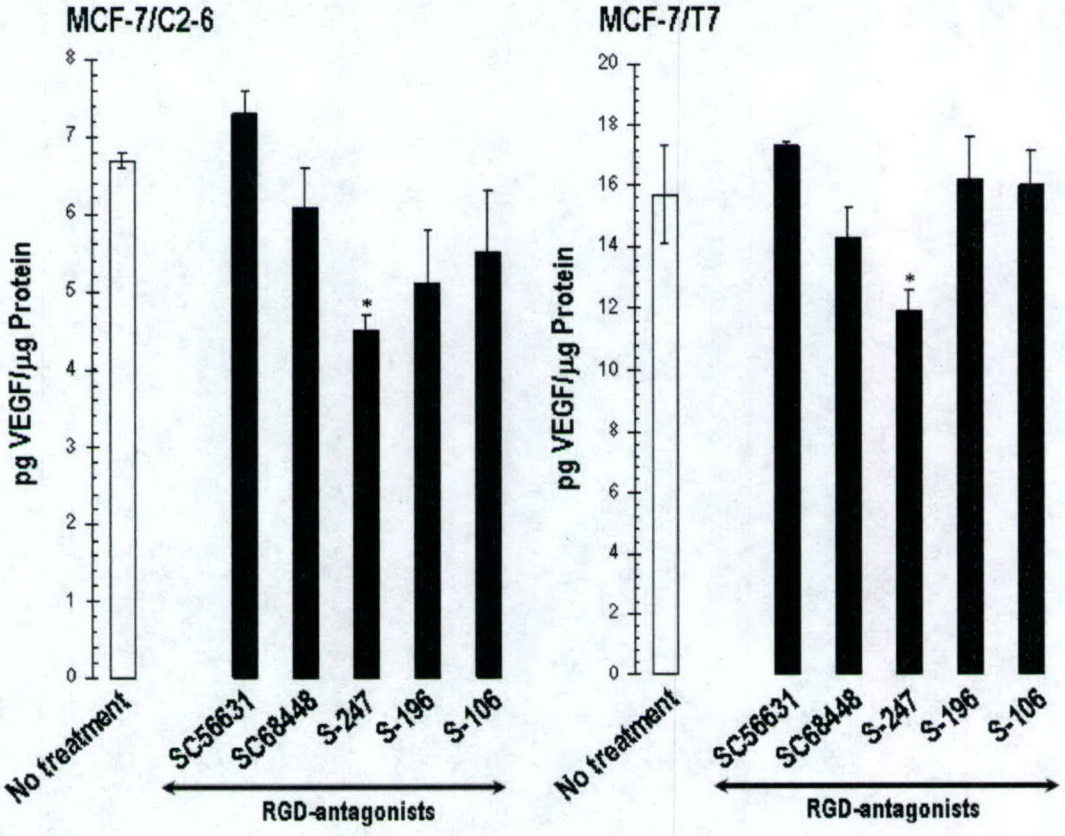


Figure 7. MCF-7 cells stably transfected to overexpress CYR61 (***left***) or HRG (***right***) were treated with $\alpha_v\beta_3$ antagonists SC56631, SC68448, S-247, S-196 and S-106 at 1 µM in 0.1% FBS-IMEM. Following 48 h treatment, the level of $VEGF_{165}$ in the supernatant was determined using a $VEFG_{165}$-specific ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in untreated cells.

10. MEK1/MEK2 → ERK1/ERK2 transduction cascade is the main signaling pathway involved in CYR61-induced up-regulation of VEGF secretion in breast cancer cells. The HRG-CYR61-$\alpha_v\beta_3$ signaling network activates several signaling pathways leading to enhanced endothelial cell survival and proliferation, including PI-3'K AKT and MEK1/MEK2 ERK1/ERK2. Therefore, we examined whether downstream of $\alpha_v\beta_3$, PI-3'K AKT or MEK1/MEK2 ERK1/ERK2 pathways were actively involved in CYR61-promoted secretion of VEGF in breast cancer cells. While down-stream of PI-3'K the content of total AKT was similar in CYR61-overexpressing and control MCF-7/V cells, active AKT (P-AKT^{Ser473}) was significantly higher in CYR61 derivatives MCF-7/C2-6 and MCF-7/C2-9 (Figure 8, *left panel*). Similarly, the content of total MAPK was similar in CYR61-overexpressing and control MCF-7/V cells, whereas the activation status of ERK1/ERK2 MAPK, as monitored by immunoblotting using polyclonal antibodies recognizing the activated form of the ERK1 and ERK2 enzymes (P-ERK1/ERK2 MAPK), was significantly higher in MCF-7/C2-6 and MCF-7/C2-9 transfectants in comparison to matched control MCF-7/V cells (Figure 8, *left panel*). LY294002, a specific inhibitor of the p110 catalytic subunit of PI-3'K, particularly affected PI-3'K/AKT signaling as indicated by the complete inhibition of P-AKT^{Ser473}. U0126, a noncompetitive inhibitor of the dual specificity MAPK kinases MEK1 and MEK2, the enzymes that activate MAPK, completely eliminated P-MAPK without affecting active AKT (Figure 8, *right panel*). The levels of total AKT and total MAPK were not altered by any of the inhibitors. These results strongly indicate that, down-stream of $\alpha_v\beta_3$, CYR61 overexpression concomitantly activates PI-3'K AKT and MEK1/MEK2 ERK1/ERK2 signaling cascades in breast cancer cells. Moreover, CYR61 seems to molecularly disrupt the cross-talk between these two signaling cascades at the level of *Raf* and AKT as the inhibition of PI-3'K does not lead to activation of the *Raf*-MEK-ERK cascade in CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones.

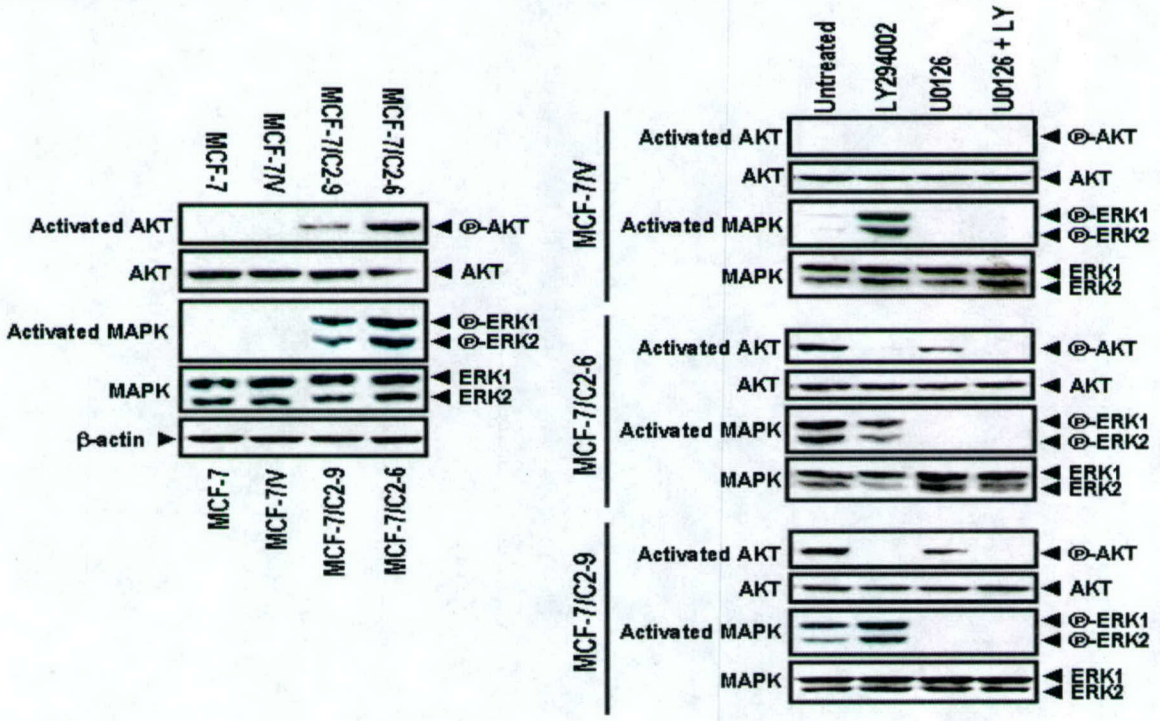


Figure 8. *Left.* Total and activated (phosphorylated) AKT and ERK1/ERK2 MAPK protein levels in CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 and matched control MCF-7/V cells. Overnight serum-starved cells at 75-80% confluence were washed two times with PBS, lysed in buffer, subjected to electrophoresis on 10% SDS-PAGE, and transferred to nitrocellulose

membranes. Immnunoblots with anti-AKT, anti-P-AKT, anti-ERK1/ERK2 MAPK, and anti-P-ERK1/ERK2 MAPK show equivalent amounts of the proteins in each extract (25 μg). Blots were re-probed with an antibody for β-actin to control for protein loading and transfer. Results are representative of three independent experiments. *Right.* Overnight serum-starved cells at 75-80% confluence were treated for 2 h with vehicle (*v/v*), LY294002 (40 μM), U0126 (20 μM), or both LY294002 and U0126. Total protein (25 μg) was resolved by SDS-PAGE and subjected to immunoblot analyses for AKT, P-AKT, ERK1/ERK2 MAPK, and P-ERK1/ERK2 MAPK as described above. Blots were re-probed with an antibody for β-actin to control for protein loading and transfer. Results are representative of three independent experiments.

To link CYR61-induced activation of PI-3'K or MAPK cascades to VEGF secretion, we used nontoxic concentrations of LY294002 and U0126, specific inhibitors of PI-3'K and of MEK1/MEK2 enzymatic activities, respectively (Figure 9). Interestingly, the use of equimolar concentrations of LY294002 and U0126 revealed a more prominent involvement of CYR61-activated MEK1/MEK2 ERK1/ERK2 signaling on the maintenance of high levels of VEGF secretion in CYR61-overexpressing breast cancer cells.

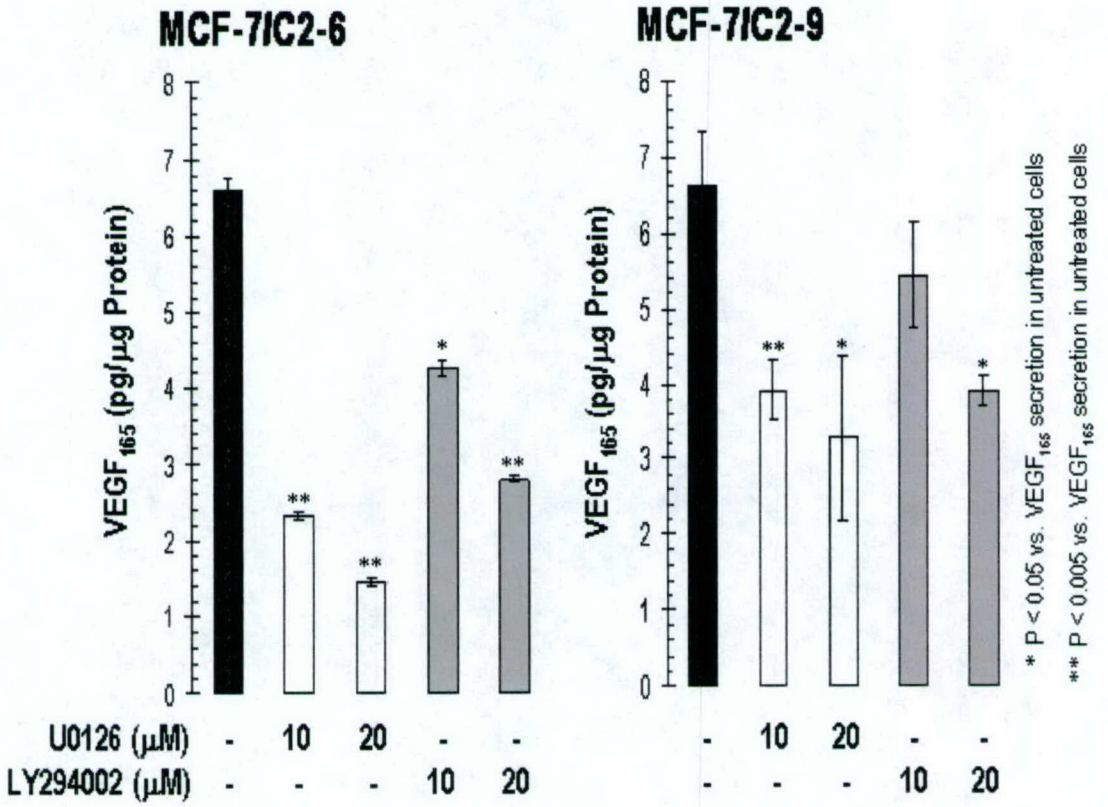


Figure 9. MCF-7 cells stably transfected to overexpress CYR61 (MCF-7/C2-6 and MCF-7/C2-9 clones) were treated with increasing concentrations of U0126 or LY294002 in 0.1% FBS-IMEM. Following 48 h treatment, the level of $VEGF_{165}$ in the supernatant was determined by the ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion levels in untreated cells

Remarkably, optimal concentrations of $\alpha_v\beta_3$ antagonists completely abolished hyperactivation of ERK1/ERK2 MAPK in both MCF-7 cells engineered to overexpress CYR61 and naturally HRG- and CYR61-overexpressing MDA-MB-231 cells, whereas the activation status of AKT did not decrease (Figure 10). These findings provide evidence that $\alpha_v\beta_3$ integrin specifically regulates cell survival, cell proliferation and $VEGF_{165}$ secretion in CYR61-overepressing breast cancer cells through activation of ERK1/ERK2 MAPK signaling, with a minor involvement of AKT activity.

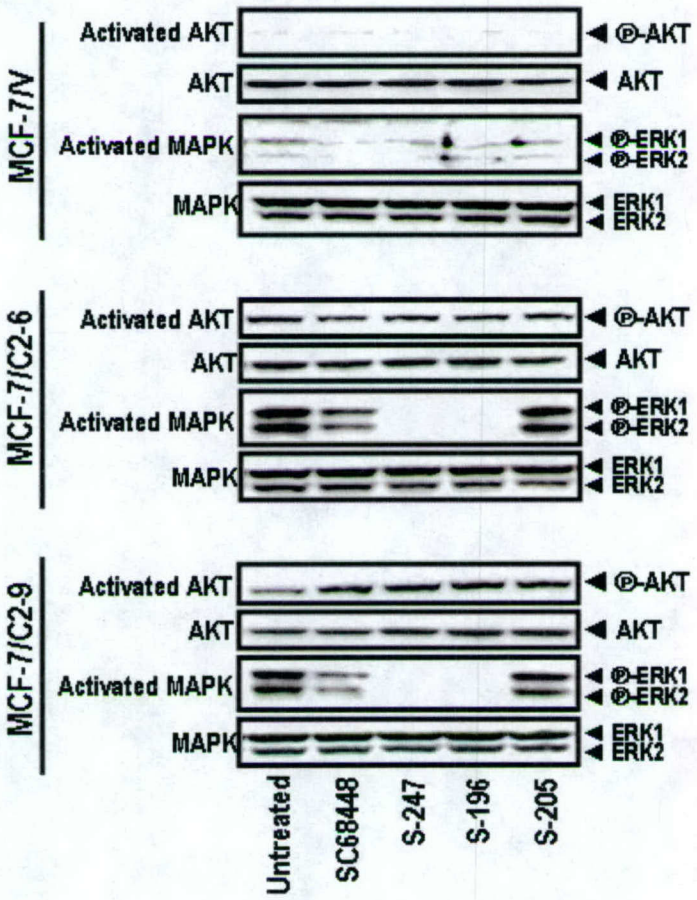


Figure 10. Functional blockade of $\alpha_v\beta_3$ integrin specifically inactivates CYR61-induced ERK1/ERK2 MAPK transduction cascade without affecting AKT activity. Overnight serum-starved MCF-7/V, MCF-7/C2-6 and MCF-7/C2-9 cells at 75-80% confluence were treated for 2 h with either vehicle (*v/v*) or 1 μM of specific $\alpha_v\beta_3$ antagonists (SC68448, S-247, S-196 or S-205). Total protein (25 μg) was resolved by SDS-PAGE and subjected to immunoblot analyses for AKT, P-AKT, ERK1/ERK2 MAPK, and P-ERK1/ERK2 MAPK as described above. Results are representative of three independent experiments

11. CYR61 overexpression induces breast cancer cell resistance to paclitaxel (Taxol™). Integrin signals are involved in diverse biological responses, including angiogenesis and tumor progression as well as in a variety of cellular activities, including cell migration, proliferation, and survival. Of interest, integrin signaling has recently been shown to modulate cancer cell responses to chemotherapeutic agents. This integrin-dependent phenomenon of innate chemoresistance has been termed cell adhesion-mediated drug resistance. Here, we evaluated whether CYR61-induced up-regulation of $\alpha_v\beta_3$, while up-regulating

$VEGF_{165}$ secretion, it would also modulate breast cancer cell response to paclitaxel (Taxol™), an antimitotic drug commonly used in the treatment of advanced or metastatic breast cancer. Using the MTT assay, which is a crude measure of cell viability, we observed that CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones were between 9 and 12-fold more resistant to Taxol-induced cytotoxicity when compared with matched control MCF-7/V cells (Figure 11*a*). We speculated that the reduced sensitivity to Taxol seen in CYR61-overexpressing MCF-7 cells was not simply the result of changes in cell proliferation, but might actually be due to a CYR61-promoted decrease in apoptotic cell death following Taxol-induced cell damage. To address this question, cells were exposed to 10 nM Taxol, cell death was measured by an ELISA that detects DNA-histone fragmentation, and the *x*–fold increase in apoptosis-related cell death was calculated by comparing the ELISA optical density readings of treated samples, with the values of the untreated cells as 1.0. Likewise, CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones treated with Taxol exhibited a negligible degree of cell death compared with that observed in control MCF-7/V cells (Figure 11*b*). Because soft-agar colony formation of cancer cells is thought to be one of the best *in vitro* correlates of the ability of cancer cells to grow *in vivo*, CYR61-overexpressing MCF-7 transfectants and MCF-7/V control cells were further analyzed for their anchorage-independent growth properties in the presence of Taxol. Very low concentrations of Taxol (< 10 nM) dramatically suppressed colony formation of empty-vector control cells growing in semisolid agar. In contrast, significant higher concentrations of Taxol (> 50 nM) were required to inhibit the anchorage-independent soft agar clonogenic growth of CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-6 clones (Figure 11*c*). Thus, under anchorage-independent growth conditions, CYR61 overexpression increased between 5 and 10 times breast cancer cell resistance to Taxol. Altogether, these findings support the notion that CYR61 plays an important role in resistance to chemotherapeutic agent-induced apoptosis and clearly demonstrate that CYR61 overexpression dramatically decreases the cytotoxic activity of Taxol towards breast cancer cells.

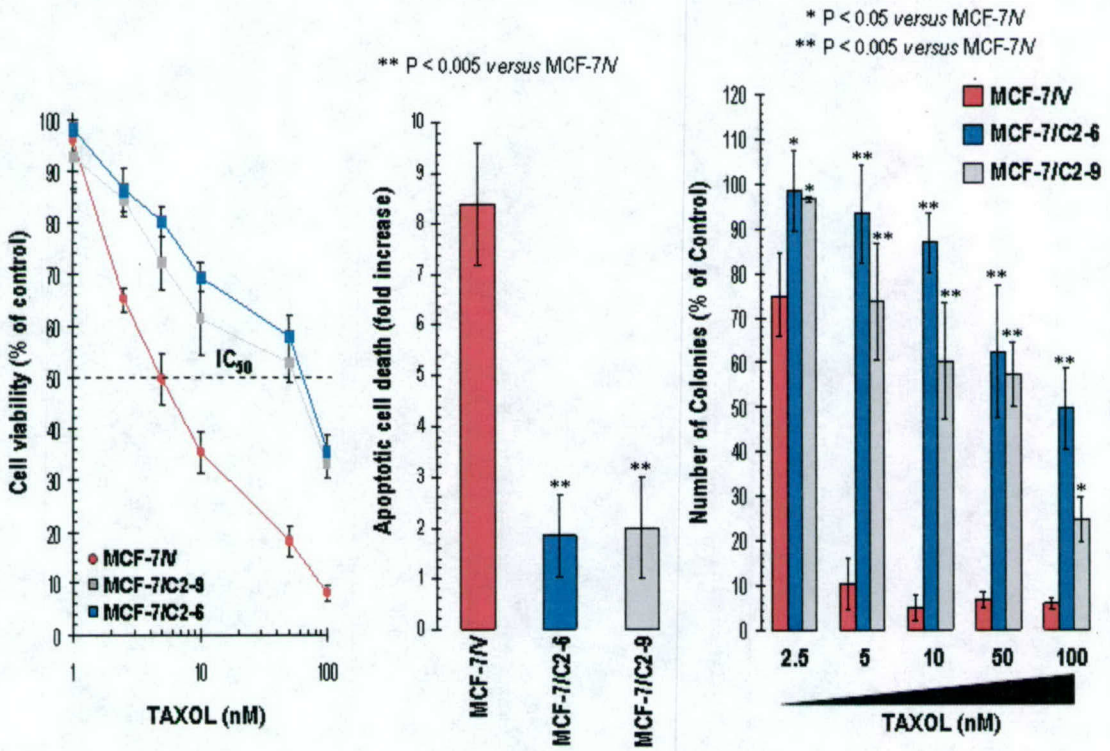


Figure 11. ***a.*** MCF-7 cells engineered to overexpress CYR61 are less sensitivity to Taxol-induced cell toxicity. CYR61-overexpressing MCF-7 cells (C2-6 and C2-9 clones) and matched control MCF-7/V cells were cultured in 96-well plates in the presence or absence of increasing concentrations of Taxol until untreated control cells reached confluence. Cell viability and IC_{50} values were then determined using a modified MTT reduction assay as described above. The data presented summarize the mean (± S.D.) of five independent experiments made in triplicate. ***b.*** CYR61 overexpression reduces Taxol-induced cell death. The induction of cell death by exposure of CYR61-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones, and matched control MCF-7/V cells to 10 nM Taxol (24 h) was assessed using the Cell Death Detection ELISA^{PLUS} kit as per manufacturer's instructions. The enrichment of histone-DNA fragments in treated cells was expressed as fold increase in absorbance as compared with control (vehicle-treated) cells. Data presented summarize the mean (*columns*) ± S.D. (*bars*) of three independent experiments made in duplicate. ***c.*** CYR61 overexpression increases breast cancer cell resistance to Taxol upon anchorage-independent conditions. Data presented summarize the mean percentages (*columns*) ± S.D. (*bars*) of five independent experiments made in triplicate (colony number obtained in untreated samples = 100%).

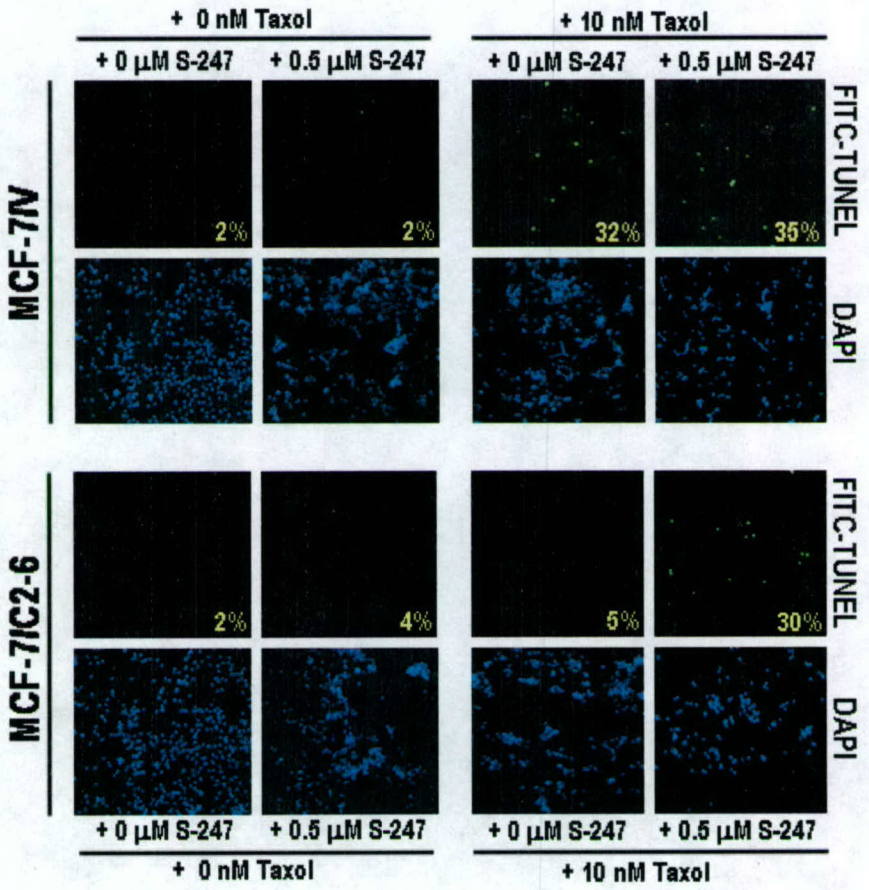
12. Functional blockade of $\alpha_v\beta_3$ synergistically enhances Taxol-induced apoptosis in CYR61-overexpressing breast cancer cells. Because apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents, we analyzed whether the failure of CYR61-overexpressing MCF-7 cells to activate apoptosis may account for CYR61-promoted resistance to Taxol. In these experiments, we employed the lowest clinically relevant concentration of Taxol (*i.e.* 10 nM) that blocks normal cell cycle progression at the G_2-M of the cell cycle and induces ERK1/ERK2 MAPK activation.

To evaluate Taxol-related apoptosis, we used a fluorometric TUNEL assay, which measure the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). In our experiments, fluorescein-12-dUTP-labeled DNA in Taxol-treated cells was visualized directly by fluorescence microscopy. With this protocol, Taxol by itself was found to induce a significant increase in basal apoptosis (*e.g.*, *versus* untreated cells) in CYR61-negative MCF-7/V cells (Figure 12, *top panels*). Conversely, the CYR61 derivative MCF-7/C2-6 did not undergo major apoptosis after Taxol exposure as assessed by TUNEL labeling (Figure 12, *bottom panels*). Single treatment with the $\alpha_v\beta_3$ antagonist S-247 of CYR61-overexpressing MCF-7/C2-6 and matched control MCF-7/V cells did induce a negligible increase in apoptotic cell death. Accordingly, we recently demonstrated that S-247 significantly reduces S-phase cell sub-population without promoting apoptotic cell death of HRG- and CYR61-overexpressing MDA-MB-231 breast cancer cells (45).

These results, altogether, strongly suggest that $\alpha_v\beta_3$ antagonists such as S-247 may specifically regulate breast cancer cell cycle progression without affecting breast cancer cell death. Interestingly, the concurrent administration of S-247 and Taxol exerted little effects on Taxol-mediated apoptosis in CYR61-negative MCF-7/V cells, whereas a significant enhancement of Taxol-induced apoptosis towards CYR61-overexpressing MCF-7/C2-6 cells was detected in the presence of the $\alpha_v\beta_3$ antagonist S-247. Indeed, the combination treatment in MCF-7/C2-6 cells produced a ~ 3-fold enhancement of apoptosis over the expected additive effect. This trend was also observed following co-exposure of CYR61 transfectants to Taxol and $\alpha_v\beta_3$ antagonists SC68448 and S-196 (data not shown). Therefore, it is tempting to postulate that CYR61 overexpression in breast cancer cells may drive breast cancer cell survival and chemoresistance by emitting a proliferative and/or survival input *via* the integrin receptor $\alpha_v\beta_3$, which might integrate signals from CYR61 to proliferative and/or anti-apoptotic signaling pathways.

Figure 12. Functional blockade of $\alpha_v\beta_3$ synergistically enhances Taxol-induced apoptotic cell death in CYR61-overexpressing MCF-7 breast cancer cells. Detection of apoptosis in cells treated with 10 nM Taxol in the absence or presence of 0.5 μM S-247 for 24 h was assessed by terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick and labeling (TUNEL) analysis was performed using the DeadEnd™ Fluorometric TUNEL System as described in "Material and methods". The immunofluorescence photomicrographs of cells undergoing apoptosis (*green staining*) and the corresponding DAPI counterstained photomicrographs

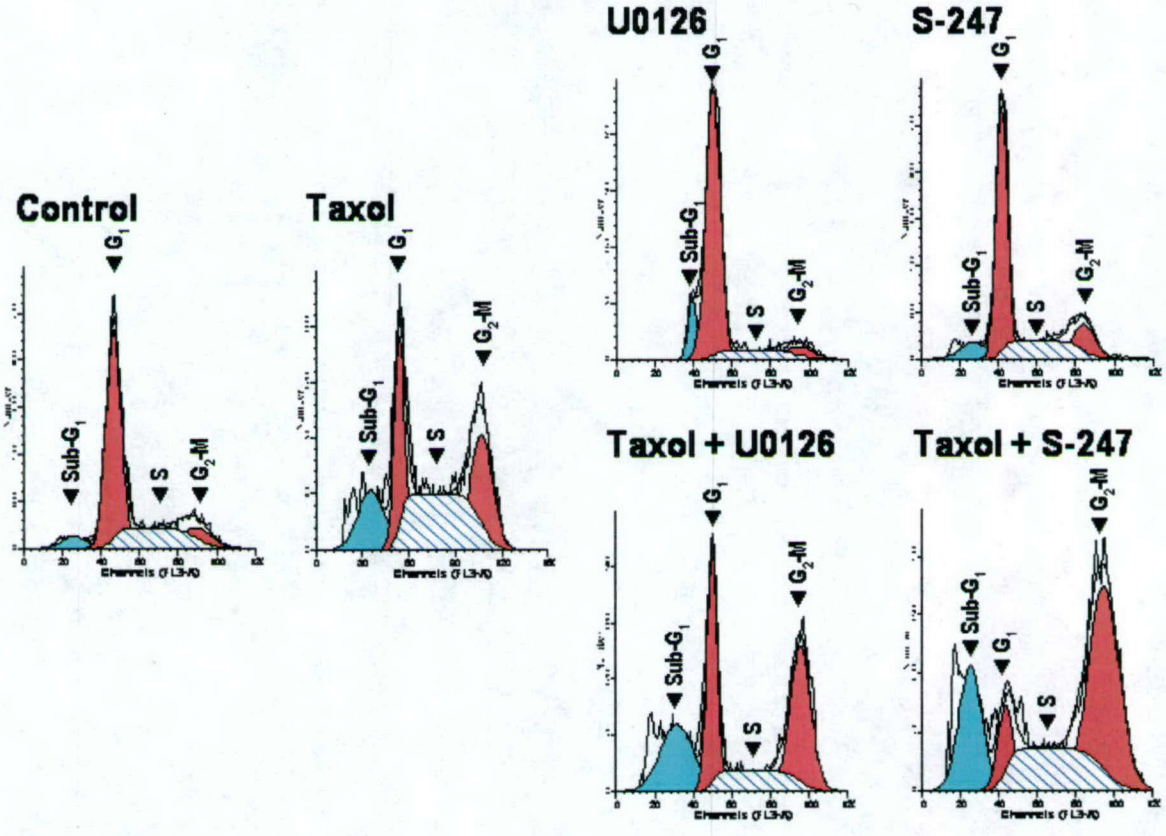
are shown. The number in the *lower right* of each panel represents the percentage of TUNEL-positive cells from one representative experiment.



13. $\alpha_v\beta_3$ antagonists regulate CYR61-overexpressing breast cancer cell survival and chemoresistance through modulation of the MEK1/MEK2 → ERK1/ERK2 MAPK pathway. As previously shown in Figure 10, $\alpha_v\beta_3$ antagonists such as S-247 and S-196 specifically block the CYR61-triggered "CYR61-$\alpha_v\beta_3$ autocrine loop" by inactivating the MEK1/MEK2 ERK1/ERK2 signaling cascade without affecting the anti-apoptotic activity of AKT. Taxol binds to β-tubulin, stabilizes the microtubule, prevents its depolymerization, leads to arrest of cells in G_2-M and ultimately, and triggers apoptosis. Interestingly, Taxol also induces the activation of the *Raf*-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway, which is considered a proliferation and cell survival pathway. Accordingly, it has been shown that inhibition of MEK combined with Taxol induces a dramatic enhancement of apoptosis in various tumor cell lines. Considering the ability of $\alpha_v\beta_3$ antagonists to specifically block ERK1/ERK2 MAPK hyperactivation in CYR61-overexpressing breast cancer cells, we reasoned that a functional blockade of $\alpha_v\beta_3$ integrin may mimic the enhancing effects of pharmacological blockers of MEK on Taxol-induced apoptotic cell death.

To examine the mechanism of enhanced apoptosis observed with Taxol and $\alpha_v\beta_3$ antagonists, their effects on cell cycle progression were studied in parallel to those induced by the pharmacological MERK/ERK inhibitor U0126 (Figure 13). First, the CYR61-overexpressing MCF-7/C2-9 clone was treated

with Taxol, S-247, and U0126 as single agents. As expected, U0126-induced inhibition of produced a significant G_2 block. 13% of the cells underwent apoptosis in the presence of Taxol, whereas a negligible increase in cell death was detected in the presence of U0126 when compared with the untreated controls. The combination of Taxol and U0126 substantially increased cell death as evidence by accumulation of a sub-G_1 population that has <2N DNA and represents dead cells. In addition, a significant increase in the percentage of cells in G_2-M was observed when MCF-7/C2-9 cells were concurrently treated with U0126 and Taxol. Similarly to U0126, a sub-optimal concentration of the $\alpha_v\beta_3$ antagonist S-247 (0.25 μM) caused little apoptosis (8% compared with 5% in untreated control cells), while it significantly decreased S-phase cell subpopulation from 31% to 13%. A more dramatic reduction in the S-phase proliferating fraction of MCF-7/C2-9 cells (up to 75% reduction at 1 μM S-247) with a modest increase in sub-G_1 apoptotic cells was observed in the presence of higher concentrations of S-247 (data not shown). Remarkably, there was a synergistic increase in the proportion of sub-G_1 cells (up to 27%), while the percentage of Taxol-treated MCF-7/C2-9 cells in G_2-M dramatically increased from 30% to 57% following co-exposure to S-247 (Figure 13). These results not only confirm that $\alpha_v\beta_3$ antagonists specifically regulate cell cycle progression without affecting apoptotic cell death but further demonstrate that functional blockade of $\alpha_v\beta_3$ signaling in CYR61-overexpressing breast ancer cells mimics the molecular effects of MEK1/MEK inhibitors by synergistically promoting both the proportion of cells in the G_2-M phase of the cell cycle and the appearance of sub-G_1 hypodiploid cells caused by Taxol (Figure 13).



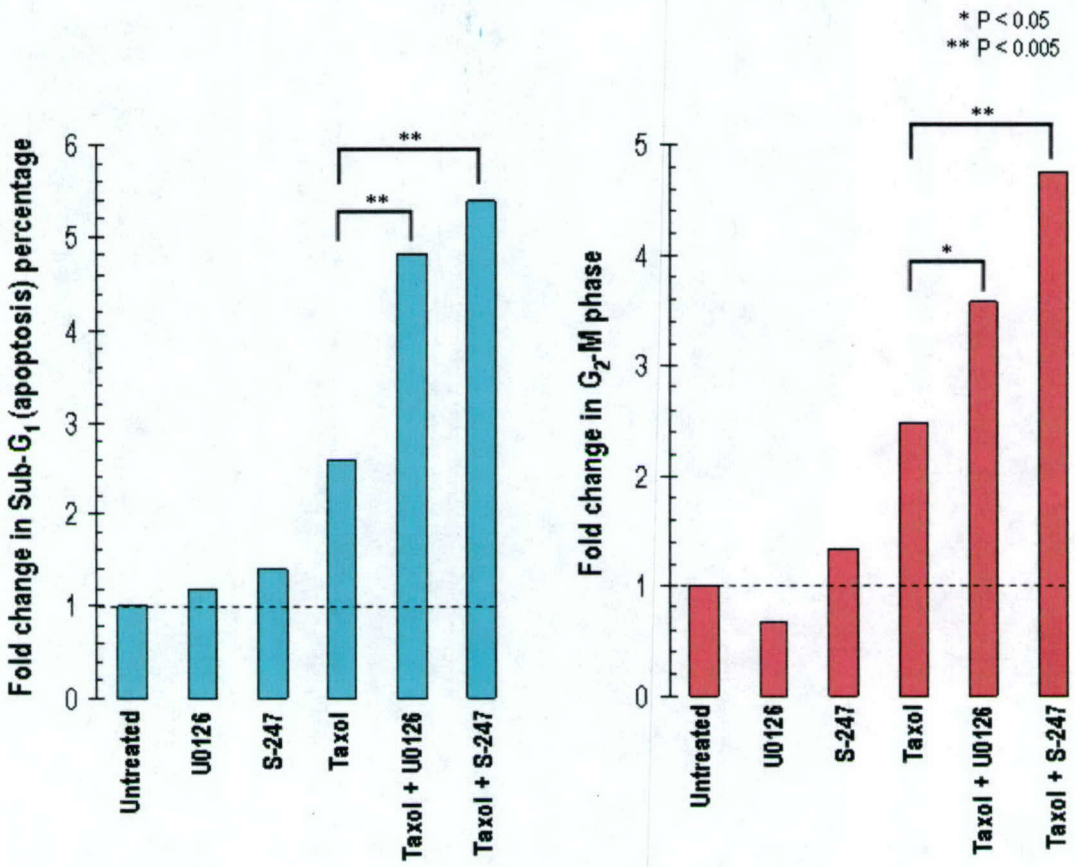


Figure 13. Blockade of $\alpha_v\beta_0$ integrin signaling by S-247 and blockade of ERK1/ERK2 MAPK activity by U0126 similarly modulate Taxol-induced effects on cell cycle progression in CYR61-overexpressing MCF-7/C2-9 cells. ***Top panels.*** Distribution of MCF-7/C2-9 cells in the different cell cycle compartments was analysed by flow cytometry after 24 h in presence of Taxol (45 nM), U0126 (20 μM), and S-247 (0.25 μM) as single agents or following concurrent combinations of Taxol *plus* U0126 or Taxol *plus* S-247. Representative cell cycle profiles are shown for each treatment. ***Bottom panels.*** The fold-change in the percentage of sub-G_1 (left panel) and G_2-M (right panel) phase MCF-7/C2-9 cells relative to untreated cells is shown on the Y-axis. Cell cycle analyses were repeated at least three times.

14. Blockade of CYR61 expression decreases cell survival and chemoresistance in HRG-overexpressing breast cancer cells. In a final scenario, we characterized breast cancer cell survival and chemosensitivity following blockade of CYR61 expression in MCF-7 cells engineered to overexpress HRG. Conditioned media from CYR61/AS-4 and CYR61/AS-6 were collected and the expression levels of CYR61 were determined by Western blotting. T7/CYR61-AS4 and T7/CYR61-AS7 clones expressed very low levels of CYR61 when compared to HRG-overexpressing MCF-7/T7 cells (Figure 14, *left panel*). In addition, blockade of CYR61 expression in MCF-7 cells engineered to overexpress HRG was able to reduce $\alpha_v\beta_3$ expression to the basal level observed in the wild type or MCF-7/V control cells (data not shown). Moreover, the levels of HRG-induced transactivation of $p185^{Her\text{-}2/neu}$ were unchanged in antisense-CYR61 transfectants when compared with those found in T7 and T7/AC-V controls. Thus, this cellular system exhibits an intact HRG-Her-2/*neu* signaling in the absence of CYR61 overexpression. Since the colony-forming assay in soft-agar measures both anchorage-independent proliferation and cell survival, we next focused on an active role of CYR61 on HRG-enhanced breast cancer cell survival. Indeed, the acquisition of anchorage-independent growth is generally considered to be one of the *in vitro* properties associated

with the malignancy of cells, and colonization of metastatic tumor cells at a distant site may be partially modeled in soft agar assays. More importantly, it is well established that MCF-7 cells are not anchorage-independent in the absence of estradiol (E_2). Colony formation of MCF-7 cells in soft agar observed, if any, represent the background level for the colony formation assay. MCF-7, MCF-7/T7, T7/CYR61-AS4, and T7/CYR61-AS7 cells were cultured in E_2-depleted media and assayed in a soft-agar assay in the absence of E_2, using phenol red-free medium containing 10% charcoal-stripped calf serum. The cells were allowed to form colonies for 2 weeks and were counted using a colony counter. As expected, MCF-7 cells did not form colonies in the absence of E_2. Interestingly, E_2-independent soft agar colony formation of HRG-overexpressing MCF-7/T7 cells was drastically reduced in the absence of high levels of CYR61 expression. Thus, T7/CYR61-AS4 and T7/CYR61-AS7 clones were almost unable to growth in an anchorage-independent manner (Figure 14, *left panel*). Moreover, down-regulation of CYR61 expression in HRG-overexpressing T7 cells significantly decreased HRG-promoted resistance to Taxol (Figure 14, *right panel*). These findings demonstrate that a functional CYR61-$\alpha_v\beta_3$ loop is *necessary* for the maintenance of HRG-enhanced cell survival and chemoresistance in breast cancer cells.

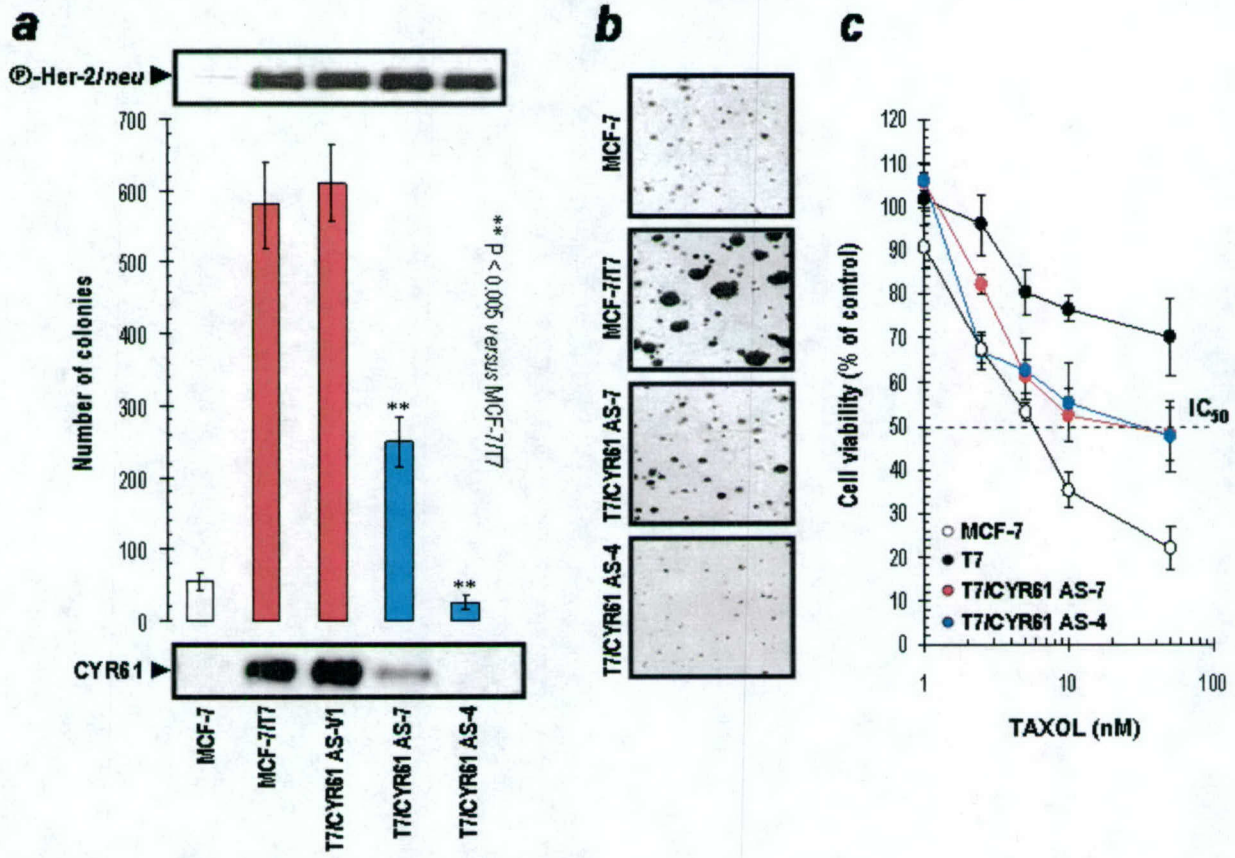


Figure 14.. ***a***. Down-regulation of CYR61 expression abolishes HRG-promoted estrogen (E_2)-independent breast cancer cell growth in anchorage-independent conditions, whereas it does not modify the activation status of Her-2/*neu*. *Top panel*. Overnight serum-starved cells at 75-80% confluence were harvested in lysis buffer, 40 μg of total protein was resolved by 3-8% Tris-Acetate NuPAGE, and subjected to immunoblot analyses for P-p185$^{Her\text{-}2/neu}$ using a Her-2/*neu* phosphorylation state specific antibody. *Middle panel*. Soft-agar (anchorage-independent) colony formation assays upon E_2-depleted conditions for MCF-7, MCF-7/V, MCF-7/T, MCF-7/T7 CYR61 AS-4, and MCF-7/T7 CYR61 AS-7 cells. The data presented are mean of number of colonies (*columns*) ± S.D. (*bars*). All assays were performed at least three times in triplicate. ***b***. Microphotographs of representative soft-agar assays are shown (150-fold magnification). *Bottom panel*. CYR61 protein levels in the conditioned media from were analyzed by Western blotting as described in Figure 1*c*. ***c***. Down-regulation of CYR61 expression reduces

HRG-enhanced breast cancer cell resistance to Taxol-induced cytotoxicity. Taxol sensitivity in T7 cells was determined using a standard colorimetric MTT reduction assay as described above. Data presented summarize the mean (± S.D.) of three independent experiments made in triplicate.

Among several angiogenic factors (VEGF, VEGF-related protein, VEGF-B, -C, -D, -E, placenta growth factor, and basic and acidic fibroblast growth factor), VEGF has been shown to be the major tumor angiogenic factor. VEGF can stimulate endothelial cell mitogenesis, and its overexpression has been detected in tumor cells, and was associated with high metastatic potential. Additionally, inhibition of VEGF signaling has been shown to impair tumor growth and suppress tumor metastasis. The present study was designed to examine the HRG-dependent regulation of the VEGF secretory isoform, $VEGF_{165}$, in breast cancer cells that not overexpress the Her-2/*neu* oncogene. MCF-7 human breast cancer cells engineered to overexpress HRG offer the distinct advantage of studying ligand-induced Her-2/*neu* receptor activation in the absence of Her-2/*neu* overexpression. In this regard, our current analysis explored a uniquely different mechanism through ligand (HRG)-mediated transactivation of Her-2/*neu* signaling and up-regulation of $VEGF_{165}$ secretion. We used matched MCF-7 parent and HRG-transfected daughter human breast cancer cells, which differ in their HRG expression level, to evaluate the role of HRG on VEGF-related breast cancer angiogenesis. In addition, we performed parallel studies in retroviral HRG-infected MCF-7 cells to circumvent the possibility that phenomena due to effects other than HRG overexpression (*i.e.* clone specific) might be observed. Using this approach, we were able to directly compare $VEGF_{165}$ secretion in Her-2/*neu*-negative parental cells with low-expression of HRG to identical daughter cells with high-expression of HRG *in vitro*.

Using a panel of breast cancer cell lines with constitutive Her-2/*neu* overexpression, HRG overexpression or engineered to stably overexpress HRG, we found that $VEGF_{165}$ secretion was significantly higher in HRG-overexpressing cells than in Her-2/*neu*-overexpressing and HRG-negative breast cancer cells. From the mechanistic point of view, it is clear that $VEGF_{165}$ secretion in HRG-overexpressing MCF-7 cells is not dependent upon Her-2/*neu* overexpression. In fact, the current data demonstrate a significant down-regulation of Her-2/*neu* protein levels following forced expression of HRG in MCF-7 cells. Thus, our results strongly suggest that HRG-induced transactivation of Her-2/*neu* signaling is *sufficient* to determine up-regulation of $VEGF_{165}$ secretion in breast cancer cells in the absence of Her-2/*neu* overexpression. We also provide evidence that the pro-angiogenic functions of HRG can be attributed to different structural domains of the protein. We demonstrate that the deletion of both the N-terminus sequences (a putative nuclear localization signal –NLS-) and the cytoplasmic domain of HRG protein in the HRG-M4 structural mutant completely abolish the up-regulation of $VEGF_{165}$ secretion promoted by the full-length HRG. Of note, we recently reported that HRG-M4 blocks the aggressive phenotype that has been associated to with the full-length HRG (44). Since the HRG-M4 protein is sequestered into a cellular compartment and is not secreted into the culture media, thus preventing its autocrine action and $p185^{Her\text{-}2/neu}$ phosphorylation, the data derived from MCF-7/HRG and MCF-7/HRG-M4 cells strongly suggest that HRG-dependent transactivation of Her-2/*neu* signaling is sufficient to induce a prominent up-regulation of $VEGF_{165}$ in human breast cancer cells. On the other hand, the deletion of the N-terminus sequences in the HRG-M1 mutant is not sufficient to reverse HRG-induced over-secretion of $VEGF_{165}$, likely because this mutant, similarly to the full-length HRG, does not deprive breast cancer cells of pro-angiogenic transduction pathways provided by the activation of Her-2/*neu* signaling. Moreover, the high levels of $VEGF_{165}$ secretion on MDA-MB-231 breast cancer cells, a natural model of HRG overexpression, were significantly diminished by transfection with antisense HRG cDNA. Interestingly, we recently demonstrated that blockade of HRG overexpression did suppress the aggressive phenotype of MDA-MB-231 breast cancer cells by inhibiting cell proliferation, preventing anchorage-independent cell growth, and suppressing the invasive potential of these cells *in vitro* (26). More importantly, we observed a

marked reduction in tumor formation, tumor size, and a lack of metastasis *in vivo* (26). From our current observations, it is reasonable to suggest that blockade of HRG expression inhibits tumorigenicity and abolishes the metastatic process by perhaps inhibiting a large cascade of molecular events, including the secretion of the angiogenic factor $VEGF_{165}$.

Since CCN1 (CYR61) is also induced in HRG-overexpressing breast cancer cells (5), and HRG promotes tumorigenicity in part *via* up-regulation of CYR61 (5), we further investigated whether CCN1 overexpression by itself was sufficient to bypass the requirement of Her-2/*neu* activation for HRG-enhanced secretion of $VEGF_{165}$. Forced expression of CCN1 in Her-2/*neu*- and HRG-negative MCF-7 human breast cancer cells resulted in a significant increase in the secretion of $VEGF_{165}$, and this association occurred in the absence of Her-2/*neu* activation. Therefore, CYR61 is *sufficient* to up-regulate breast cancer cell secretion of $VEGF_{165}$ in the absence of HRG and/or Her-2/*neu* overexpression in breast cancer cells. Interestingly, CYR61 overexpression in HRG- and Her-2/*neu*-negative MCF-7 cells was accompanied by activation of MAPK and PI-3'K/AKT signaling pathways. Inactivation of the MAPK signaling using the specific inhibitor U0126 completely prevented CYR61-induced secretion of $VEGF_{165}$. Pharmacological inhibition of PI-3'K activity partially reversed CYR61-stimulated $VEGF_{165}$ secretion. These results indicate that, down-stream of CYR61, MAPK and PI-3'K-dependent AKT activity are participating in the transduction of signals that result in the increased secretion of $VEGF_{165}$ found in CYR61-overexpressing MCF-7 cells. Moreover, these observations suggest that Her-2/*neu*, HRG, and CYR61 share similar signaling pathways, which may also account for the biological effect of CYR61 on the promotion of more aggressive breast cancer phenotypes. Interestingly, S-247, a potent antagonist of CYR61 receptor $\alpha_v\beta_3$ integrin, significantly decreased CYR61-induced secretion of $VEGF_{165}$ in MCF-7/CYR61 and MCF-7/HRG transfectants. The incubation of MCF-7/CYR61 transfectants with S-247 did not decrease the activation status (*i.e.*, phosphorylation) of AKT. However, the functional blockade of $\alpha_v\beta_3$ integrin receptor completely abolished MAPK hyperactivation in MCF-7 cells engineered to overexpress CCN1 or HRG. Because integrins are involved in numerous pathways, S-247 is likely to inhibit $\alpha_v\beta_3$-dependent cellular signaling *via* MAPK but independently of AKT. Although the exact mechanism(s) by which CYR61 enhances HRG-stimulated $VEGF_{165}$ secretion is still unknown, it is tempting to postulate that CCN1-induced activation of $\alpha_v\beta_3$-MAPK signaling may represent a previously unrecognized Her-2/*neu*-independent pathway involved in this phenotype. Importantly, the antisense down-regulation of CYR61 expression significantly decreased $VEGF_{165}$ secretion in HRG-overexpressing MCF-7/T7 transfectants. These results strongly suggest that a CYR61-dependent signaling through $\alpha_v\beta_3$ integrin may be *necessary* for the maintenance of high levels of $VEGF_{165}$ secretion in HRG-overexpressing human breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- High levels of HRG, independently of Her-2/*neu* overexpression, leads to up-regulation of $VEGF_{165}$ secretion in human breast cancer cells.

- HRG-stimulated secretion of $VEGF_{165}$ requires an autocrine action of HRG on Her-2/*neu*-dependent signaling. However, HRG-stimulated secretion of $VEGF_{165}$ does not require a nuclear localization of HRG.

- The growth factor-inducible immediate-early gene CYR61, a down-stream effector of HRG-induced breast cancer progression, is *sufficient* to induce up-regulation of $VEGF_{165}$ in human breast cancer cells in the absence of HRG and Her-2/*neu* overexpression.

- CYR61 enhances $VEGF_{165}$ secretion *via* activation of MAPK and PI-3'K/AKT signaling cascades.

- CYR61 is *necessary* for the maximal induction of HRG-dependent secretion of $VEGF_{165}$ in human breast cancer cells.

- The activation of a CYR61/$\alpha_v\beta_3$/MAPK signaling network could drive $VEGF_{165}$ secretion in CYR61- and HRG-overexpressing human breast cancer cells.

- CYR61 is sufficient to promote breast cancer cell proliferation, cell survival, and Taxol resistance through a $\alpha_v\beta_3$-activated MAPK signaling.

- The identification of a "CYR61-$\alpha_v\beta_3$ autocrine loop" in the epithelial compartment of breast carcinoma strongly suggests that targeting $\alpha_v\beta_3$ may simultaneously prevent breast cancer angiogenesis, growth, and chemoresistance

CONCLUSIONS

Depending on the biological context and cell model system, CYR61 is thought to act in an autocrine-paracrine manner to promote cell growth, migration, and angiogenesis, through its interaction with integrin receptors. Remarkably, most of the CYR61-promoted effects are mediated *via* its direct binding with the integrin receptor $\alpha_v\beta_3$. We recently postulated that CYR61-enhanced breast cancer progression may require a concomitant overexpression of $\alpha_v\beta_3$. Indeed, we observed that $\alpha_v\beta_3$ levels were significantly augmented in MCF-7 cells engineered to overexpress HRG when compared with control cells. In addition, we determined that functional blockade of $\alpha_v\beta_3$ using LM609, a monoclonal antibody which binds to a conformational epitope resulting from the post-translational association of the α_v and β_3 subunits, completely blocked the Matrigel outgrowth of HRG-overexpressing breast cancer cells. More recently, we provided the first indication showing that up-regulation of $\alpha_v\beta_3$ integrin expression in human breast epithelial cells can be achieved solely by CYR61 overexpression, irrespective of HRG status, while CYR61 appears to be the molecular connector between HRG and $\alpha_v\beta_3$ overexpression in breast cancer cells. These results strongly suggest that a functional CYR61-triggered $\alpha_v\beta_3$ signaling is required for maintaining the invasive capacity of breast cancer cells overexpressing HRG. Moreover, it is reasonable to hypothesize that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, through the interaction of CYR61 with its integrin receptor $\alpha_v\beta_3$.

The results arising from this proposal provide new insights for additional understanding of the role of HRG and CYR61 in breast cancer progression. Several oncogenes, growth factors, hormones and hypoxia have been shown to up-regulate VEGF expression, an angiogenic factor of reference. We previously demonstrated that CYR61 is differentially expressed in breast cancer cells overexpressing HRG, a member of the epidermal growth factor-like growth factor family that regulates angiogenesis *via* up-regulation of the expression and secretion of VEGF. More recently, we found that CYR61-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of VEGF. CYR61 stimulates tumor vascularization by acting as an angiogenic inducer of endothelial cells, as VEGF does. Considering that CYR61 is an angiogenic ligand for $\alpha_v\beta_3$ integrin receptor in endothelial cells, CYR61 might mediate breast cancer angiogenesis in a paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor. Furthermore, our current results support the notion that the up-regulatory actions of CYR61 on the secretion of VEGF are not restricted to the endothelial compartment of breast carcinomas, but also occur, in an autocrine-dependent manner, in the epithelial compartment. Interestingly, it seems that CYR61 can drive VEFG secretion in both cellular compartments *via* the integrin receptor $\alpha_v\beta_3$.

Altogether, these findings strongly suggest that a CYR61-$\alpha_v\beta_3$-regulated pro-metastatic signaling may occur in breast carcinomas. Since CYR61 overexpression by itself activates the expression of its own $\alpha_v\beta_3$ integrin receptor, up-regulation of CYR61 expression in the epithelial compartment of breast carcinoma may coordinate breast tumorigenesis and malignant progression in several concerted modes (Figure 15): 1) by coordinating breast tumor epithelial cell migration as a chemokinetic factor; 2) by promoting breast cancer epithelial cell proliferation in an autocrine/paracrine fashion either augmenting the bioactivity of other growth factors; 3) by enhancing breast cancer epithelial cell survival and chemoresistance through activation of pro-survival signaling pathways (*i.e.*, ERK1 ERK2 MAPK and/or PI-3'K AKT) down-stream of $\alpha_v\beta_3$; 4) by regulating endothelial cell survival and recruitment during tumor neovascularization in a paracrine fashion through an $\alpha_v\beta_3$-dependent mechanism; and 5) by synergistically enhancing HRG-stimulated secretion of VEGF *via* $\alpha_v\beta_3$ integrin receptor in breast epithelial cells. From a clinical perspective, our description of a novel CYR61-triggered "CYR61-$\alpha_v\beta_3$ autocrine loop" in breast epithelial cancer cells

capable to regulate cell proliferation, survival, invasion, chemosensitivity and VEGF secretion, together with the cytotoxic, chemosensitizing and anti-VEGF effects of small peptidomimetics antagonists of $\alpha_v\beta_3$, strongly suggest that current and future antagonists of specific integrins, such those used in this study directed against $\alpha_v\beta_3$, or more specific anti-HRG and anti-CYR61 strategies, may have the potential to suppress tumorigenicity and metastasis of HRG- and CYR61-overexpressing breast carcinomas by decreasing VEGF-dependent breast cancer angiogenesis.

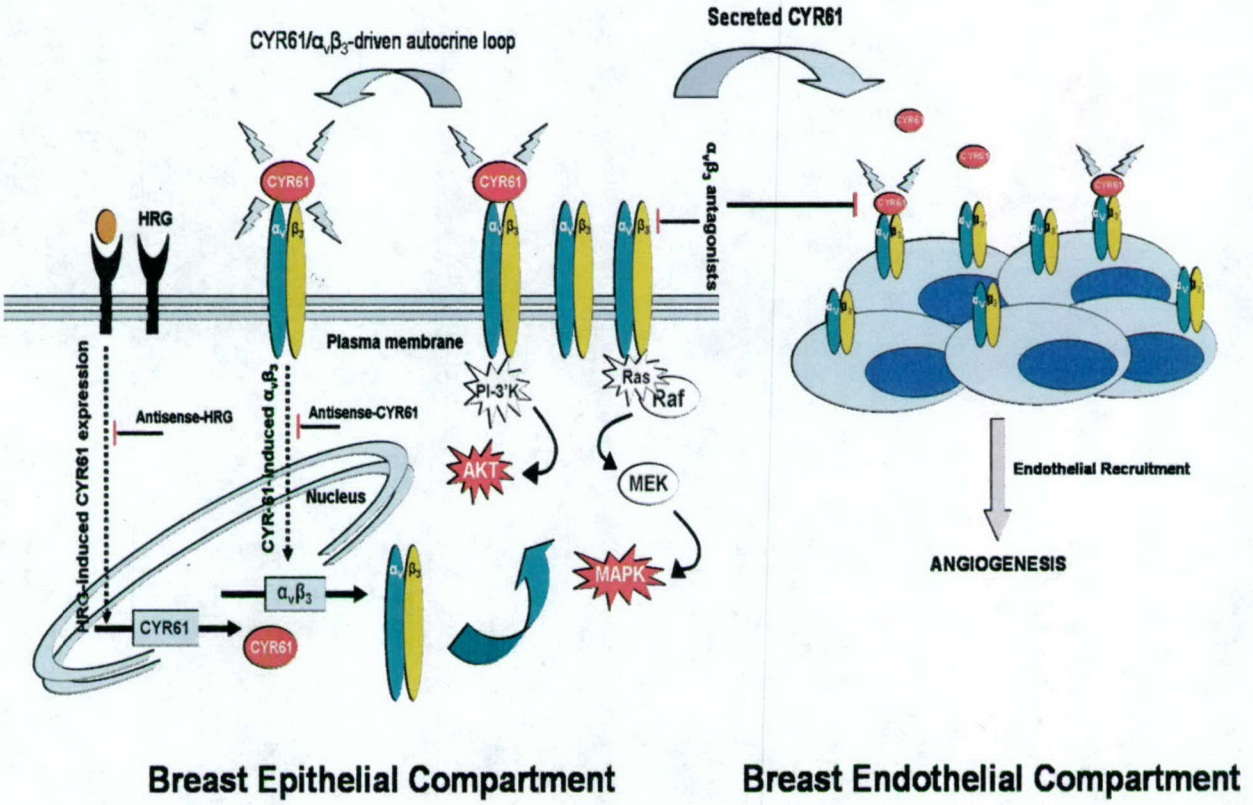


Figure 15. HRG-induced up-regulation of CYR61 may predispose breast tumor epithelial cells toward deregulated proliferation and chemoresistance: A working model. The functional blocking of $\alpha_v\beta_3$ integrin differentially induces cytotoxicity towards HRG- and CYR61-overexpressing breast cancer cells, thus suggesting that a CYR61-activated $\alpha_v\beta_3$ integrin signaling is actively involved in breast cancer cell survival. Since CYR61 overexpression by itself is sufficient to activate $\alpha_v\beta_3$ integrin expression, up-regulation of CYR61 in breast cancer epithelial cells may coordinate a metastatic phenotype in an autocrine manner by activating $\alpha_v\beta_3$-downstream signaling cascades such as PI-3'K AKT and MEK1/MEK ERK1/ERK2 MAPK, which, in turn, promotes both the up-regulation of VEGF secretion and the acquisition of a chemotherapy-resistant breast cancer phenotype. From a clinical perspective, our description of a novel CYR61-triggered "CYR61-$\alpha_v\beta_3$ autocrine loop" in breast epithelial cancer cells, together with the cytotoxic, chemosensitizing, and anti-VEGF effects of small peptidomimetic antagonists of $\alpha_v\beta_3$ towards CYR61- and HRG-overexpressing breast cancer epithelial cells, provide a starting point to further evaluate the use of agents such as integrin antagonists that should permit a potentially synergistic strike against the breast tumor and its supporting vasculature.

REPORTABLE OUTCOMES

1. Grant-related Abstracts/Presentations

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and **Ruth Lupu**. "The Angiogenic Factor CYR61, a Downstream Effector of Heregulin, Protects Breast Cancer Cells from Paclitaxel-induced Cell Death Through Integrin $\alpha_v\beta_3$" Abstract #366. **European Journal of Cancer** Vol. 38, Supplement 7, p. 108. November 2002.
14th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
November 19-22, 2002, Frankfurt, Germany.

Ruth Lupu, and **Javier A. Menendez**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway". **Tumor Progression Control and Hormones (non-steroidal) Session** of the **International Congress on Hormonal Steroids and Hormones and Cancer.**
Fukuoka, Japan, October 21-25, 2002.

Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, David Griggs, and **Ruth Lupu**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway" **International Journal of Molecular Medicine.** November 2002.
7th International Meeting on Molecular Oncology
Crete, Greece, 2002.

Javier A. Menendez, Inderjit Mehmi, David Griggs, and **Ruth Lupu**. "The angiogenic factor CCN1 (CYR61) protects breast cancer cells from Taxol-induced cell death"
5th Annual Lynn Sage Breast Cancer Symposium Agenda.
September 17-21. Chicago, Illinois, 2003.

Luciano Vellon, **Javier Abel Menendez**, and **Ruth Lupu**. "Heregulin regulates alpha$_v$ beta$_3$ ($\alpha_v\beta_3$) integrin expression in breast cancer cells"
Proc. Am. Assoc. Cancer Res. Abstract # LB-77.
American Association for Cancer Research 95th Annual Meeting
March 27-31, 2004, Orlando (FL), USA.

Ruth Lupu, Luciano Vellon, and **Javier A. Menendez**. "A novel CYR61-triggered CYR61-$\alpha_v\beta_3$ integrin loop regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway"
3rd International Workshop on the CCN family of genes
October 20-23, 2004, Saint-Malo, France.

Javier A. Menendez, Luciano Vellon, and **Ruth Lupu**. "Characterization of a CYR61-triggered "CYR61-$\alpha_v\beta_3$ autocrine loop" in the epithelial compartment of breast carcinoma that regulates cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway"
6th Lynn Sage Breast Cancer Symposium
Abstract #B17
October 30, 2004, Chicago, IL, USA.

Luciano Vellon, **Javier A. Menendez**, and **Ruth Lupu**. "$\alpha_v\beta_3$ regulates Heregulin (HRG)-induced cell proliferation and survival in breast cancer"
6th Lynn Sage Breast Cancer Symposium
Abstract #B18
October 30, 2004, Chicago, IL, USA.

Luciano Vellon, **Javier A. Menendez**, and **Ruth Lupu**. "$\alpha_v\beta_3$ integrin is a novel molecular marker of breast cancer chemosensitivity"
6th Lynn Sage Breast Cancer Symposium
Abstract #B19
October 30, 2004, Chicago, IL, USA.

2. Grant-related Manuscripts

Javier A. Menendez, Inderjit Mehmi, David G. Griggs, and **Ruth Lupu**. "The Angiogenic Factor CYR61 in Breast Cancer: Molecular pathology and Therapeutic Perspectives" ***Endocrine-Related Cancer*** 10 (2): 141-152, 2003. Review.

Javier A. Menendez, Luciano Vellon, Inderjit Mehmi, Poh K. Teng, Bharvi P. Oza, Vishal A. Verma, David Griggs, and **Ruth Lupu**. "A novel CYR61-triggered "CYR61-$\alpha_v\beta_3$ loop" regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway". **ONCOGENE** 24(5): 761-779, 2005. Article.

Luciano Vellon, **Javier A. Menendez**, David G. Griggs, and **Ruth Lupu**. "$\alpha_v\beta_3$ Integrin Regulates Heregulin (HRG)-induced cell proliferation and survival in breast cancer". ***Oncogene*** (*In press*, 2005). Article.

Javier A. Menendez, Luciano Vellon, Bharvi P. Oza, and Ruth Lupu. "The angiogenic inducer CYR61 synergistically enhances Heregulin-stimulated secretion of Vascular Endothelial Growth Factor (VEGF) via $\alpha_v\beta_3$ integrin receptor: A novel therapeutic target for small peptidomimetic $\alpha_v\beta_3$ integrin antagonists". **International Journal of Molecular Medicine** (*In press*, 2005). Article.

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After this training period of the DOD BCRP post-doctoral traineeship I have developed and mastered several essential techniques in biochemistry, cell biology, and molecular biology, which is evident in the research accomplishments described above. In addition, it has offered me tremendous opportunities to establish interesting collaborations with other researchers in the field of human breast cancer. With the support from DOD, the proposed subject shall enhance our understanding at both the cellular and molecular levels of breast cancer progression, and could result in new molecular anticancer therapies based on blockade of HRG, CYR61, and/or the integrin receptor $\alpha_v\beta_3$.

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APPENDICES

Curriculum Vitae

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EDUCATION and TRAINING

Doctoral Degree (*Ph. D.*) in BIOLOGICAL SCIENCES
Project: "Fatty Acids in Breast Cancer Progression: Therapeutic Implications"
Cum Laude
Faculty of Biology
Complutense University, Madrid, Spain

October, 2001

Research Sufficiency (*M. S.*) "BIOCHEMISTRY and MOLECULAR BIOLOGY"
Faculty of Biology
Complutense University, Madrid, Spain

December, 2000

Graduate Degree (*B. S.*) in BIOLOGICAL SCIENCES
(FUNDAMENTAL BIOLOGY)
Faculty of Biology
Oviedo University, Spain (1991-1997)

March, 1997

PROFESSIONAL POSITIONS

Pre-Doctoral Fellow
Division of Medical Oncology,
Hospital Universitario 12 de Octubre
Madrid, Spain

October 1997-October 2001

Biologist Visiting Post-Doctoral Fellow
Life Science Division, Ernest Orlando Lawrence
Berkeley National Laboratory (LBNL)
University of Berkeley, California, USA

November 2001-June 2002

Post-Doctoral Fellow
Department of Medicine, Evanston Northwestern Healthcare (ENH)
Breast Cancer Translational Research Laboratory,
ENH Research Institute (ENHRI)
Evanston, Illinois, USA

June 2002-January 2003

Research Assistant Professor
Department of Medicine, Division of Hematology-Oncology,
Northwestern University Feinberg School of Medicine
Chicago, Illinois, USA

January 2003-

Research Scientist
Department of Medicine, Evanston Northwestern Healthcare (ENH)
Breast Cancer Translational Research Laboratory, ENH Research Institute (ENHRI)
Evanston, Illinois, USA

September 2004-

FELOWSHIPS and AWARDS

Fellowships

1997-2000 National Program to the Formation of Researchers in the Modality of Post-Grade Formation, National Institute of Health of Spain (INSALUD)
MINISTRY OF EDUCATION AND CULTURE (SPAIN)
Pre-Doctoral Fellowship

2001-2002 Department of Defense Breast Cancer Research Program
Post-Doctoral Traineeship
DEPARTMENT of DEFENSE (USA)
Principal Investigator

Awards

2001 Awards on Food, Nutrition and Health
DANNON INSTITUTE (SPAIN)

2004 CAREER DEVELOPMENT AWARD
Specialized Program of Research Excellence –**SPORE**- in Breast Cancer
National Cancer Institute –NCI- (USA)

PROFESSIONAL SOCIETIES

American Association for Cancer Research (AACR)
Associate Member

2002-

RELEVANT RESEARCH EXPERIENCE (PROJECTS)

- Project title: **"MOLECULAR MECHANISMS and THERAPEUTIC IMPLICATIONS of a NOVEL BI-DIRECTIONAL CROSS-TALK between the LIPOGENIC ENZYME FATTY ACID SYNTHASE (FAS) and the *HER-2/neu* ONCOGENE in BREAST CANCER"**

Sponsor: **The Susan G. Komen Cancer Foundation: Basic, Clinical, and Translational Cancer Research)**

Position: *Principal Investigator*
Center: Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA).

Funding number: BCTR0403141
Duration: 2004-2005
Level of Funding: $250,000.00
Coordinator: Dr. Javier A. Menendez.

2004

- Project title: **"CHARACTERIZATION OF FATTY ACID SYNTHASE (FAS) AS A PREDICTIVE MARKER OF BREAST CANCER SENSITIVITY TO CHEMOTHERAPY"**

Sponsor: **Department of Defense, Breast Cancer Research Program –BCRP-: Concept Awards**

Position: *Principal Investigator*

Center: Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA).

Funding number: BC033538
Duration: 2004-2005
Level of Funding: $114,000.00/Year
Coordinator: Dr. Javier A. Menendez.

2004

- Specialized Program of Research Excellence –SPORE- in Breast Cancer, National Cancer Institute –NCI-: CAREER DEVELOPMENT AWARD

Position: *Principal Investigator*
Center: Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA).

Funding number:

Duration: 2004-2005
Level of Funding: $50,000.00/Year
Coordinator: Dr. Javier A. Menendez.

2004

- Project title: **"MOLECULAR TARGETING OF TUMOR-ASSOCIATED FATTY ACID SYNTHASE: A NOVEL THERAPEUTIC APPROACH AGAINST *HER-2/NEU*-OVEREXPRESSING BREAST CARCINOMAS"**

Sponsor: **Specialized Program of Research Excellence –SPORE- in Breast Cancer, National Cancer Institute –NCI-: Pilot Projects Program**

Position: *Principal Investigator*
Center: Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA).

Funding number: PP2

Duration: 2003-2004
Level of Funding: $50,000.00/Year
Coordinator: Dr. Javier A. Menendez.

2003

- Project title: **"PHARMACOLOGICAL INHIBITION OF FATTY ACID SYNTHASE: A NEW THERAPEUTIC APPROACH in *Her-2/neu*-OVEREXPRESSING BREAST CANCER"**

Sponsor: **Evanston Northwestern Healthcare Breast Program: Concept/Pilot Award Program**

Position: *Principal Investigator*
Center: Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA).

Duration: May 2003-March 2004
Level of Funding: $50,000.00/Year
Coordinator: Dr. Javier A. Menendez.

2003

-Project title: **"ROLE OF HEREGULIN IN BREAST CANCER NEOVASCULARIZATION"**

Sponsor: **Department of Defense, Breast Cancer Research Program –BCRP-**
Position: *Principal Investigator*
Center: Life Science Division, Lawrence Berkeley National Laboratory -University of Berkeley (California, USA)-, and Department of Medicine, Evanston Northwestern Healthcare Research Institute (Evanston, Illinois, USA)
Funding Number: DAMD17-001-0305
Duration: 2001-2004
Level of Funding: $42,000.00/Year
Coordinator: Dr. Ruth Lupu.

2001-2002

- Project title: **"EXPERIMENTAL EVALUATION OF DIETARY FATTY ACIDS AS A THERAPEUTIC STRATEGY IN BREAST CANCER"**

Sponsor: **Danone Institute, Barcelona, Spain**

Position: *Principal Investigator*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Level of Funding: $25,000/Year
Coordinator: Dr. Ramón Colomer Bosch

2001

- Project title: **TAXOL® EFFICACY AND FATTY ACIDS IN BREAST CANCER"**

Sponsor: **Bristol-Myers Squibb, Spain**

Position: *Principal Investigator*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

2001

- Project title: "**CLINICAL IMPLICATIONS OF OMEGA-3 FATTY ACIDS IN BREAST CANCER PROGRESSION**"

Sponsor: **Comunidad Autonoma de Madrid, Spain**

Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

2000-2001

- Project title: **"OMEGA-3 FATTY ACIDS IN THE PROGRESSION OF BREAST CANCER"**

Sponsor: **Sociedad Espanola de Oncologia Medica –SEOM-**

Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1999-2000

- Project title: **"FATTY ACIDS MODULATION OF ANGIOGENIC FACTORS AND ONCOGENES EXPRESSION"**

Sponsor: **Fondo de Investigacion Sanitaria (FIS 96/0226)**

Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1997-1998

- Project title: **"EXPERIMENTAL EVALUATION OF OLIVE OIL IN THE PREVENTION OF BREAST CANCER METASTASIS"**

Sponsor: **Ministry of Education and Culture, Spain (OLI96/2114)**

Position: *Pre-Doctoral Fellow*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1997-2000

BIBLIOGRAPHY

A. PUBLICATIONS
(Peer-reviewed publications in chronological order)

1. **J. A. Menéndez**, M. M. Barbacid, S. Montero, E. Sevilla, E. Escrich, H. Cortés-Funes and R. Colomer. "Effect of Gamma-Linolenic Acid and Oleic Acid on Paclitaxel Cytotoxicity in Human Breast Cancer Cells" **EUROPEAN JOURNAL of CANCER** 37(3): 402-411, 2001. Regular Article.

2. **Javier Abel Menéndez**, Eduard Escrich, Montserrat Solanas and Ramon Colomer. "Olive oil: A new strategy in the treatment of breast cancer" **MERCACEI** 133-136. February 2001. (Spanish). Regular Article.

3. **Javier Abel Menendez** and Ramon Colomer. "Omega-3 Fatty Acids and Breast Cancer Progression" **SEOM BULLETIN** 20: 27-29, 2001. (Spanish). Regular Article.

4. Colomer, R., Montero, S., Ropero, S., **Menendez, J.**, Solanas, M., and Escrich, E. "The *HER-2* Oncogen as Example of Diagnostic and Therapeutic Progress in Breast Cancer" **REVISTA de SENOLOGIA y PATOLOGIA MAMARIA** Vol. 14 (1): 8-19, 2001. (Spanish). Review.

5. Solanas, M., Hurtado, A., Costa, I., Moral, R., **Menendez, J. A.**, Colomer, R., and Escrich, E. "Effects of a High Olive Oil Diet on the Clinical Behavior and Histopathological Features of Rat DMBA-induced Mammary Tumors Compared With a High Corn Oil Diet" **INTERNATIONAL JOURNAL of ONCOLOGY** 21(4): 745-753, 2002. Regular Article.

6. **J. A. Menéndez**, S. Ropero, M. M. Barbacid, S. Montero, E. Sevilla, M. Solanas, E. Escrich, H. Cortés-Funes and R. Colomer. "Synergistic Interaction Between Vinorelbine and Gamma-Linolenic Acid in Breast Cancer Cells" **BREAST CANCER RESEARCH and TREATMENT** 72 (3): 203-219, 2002. Regular Article.

7. **Javier Abel Menéndez**, Maria del Mar Barbacid, Sagrario Montero, Santiago Ropero, Eduard Escrich, Hernán Cortés-Funes and Ramon Colomer. "Effects of Dietary Fatty Acids on the Proliferation, Adhesion and Metastatic Potential of Breast Cancer Cells: An Experimental Review" **REVISTA de ONCOLOGIA** 4 (2): 77-84, 2002. Regular Article.

8. **Javier A. Menendez**, Inderjit Mehmi, David G. Griggs, and Ruth Lupu. "The Angiogenic Factor CYR61 in Breast Cancer: Molecular pathology and Therapeutic Perspectives" **ENDOCRINE-RELATED CANCER** 10 (2): 141-152, 2003. Review.

9. **Javier A. Menéndez**, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Novel Signaling Molecules Implicated in Tumor-associated Fatty Acid Synthase-dependent Breast Cancer Cell Proliferation and Survival: Role of Exogenous Dietary Fatty Acids, p53-p21$^{WAF1/CIP1}$, ERK1/2 MAPK, p27^{KIP1}, BRCA1, and NF-κB". **INTERNATIONAL JOURNAL of ONCOLOGY** 24 (3): 591-608, 2004. Regular Article.

10. **Javier A. Menéndez**, Ruth Lupu, and Ramon Colomer. "Inhibition of Tumor-associated Fatty Acid Synthase Hyperactivity Induces Synergistic Chemosensitization of *HER-2/neu*-overexpressing human breast cancer cells to Docetaxel (Taxotere®)". **BREAST CANCER RESEARCH and TREATMENT** 84 (2): 183-195, 2004. Short communication.

11. **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "ω-6 polyunsaturated fatty acid gamma-linolenic Acid (GLA; 18:3n-6) is a Selective Estrogen Response Modulator in human breast cancer cells: GLA antagonizes Estrogen Receptor (ER)-dependent transcriptional activity, transcriptionally represses ER expression, and synergistically enhances Tamoxifen and ICI 182,780 (Faslodex) efficacy in human breast cancer cells". **INTERNATIONAL JOURNAL of CANCER** 109(6): 949-954, 2004. Letters to the editor.

12. **Javier A. Menéndez**, Santiago Ropero, Ruth Lupu, and Ramon Colomer. "ω-6 Gamma-Linolenic fatty acid Enhances Docetaxel Cytotoxicity in Human Breast Carcinoma Cells: Relationship to lipid peroxidation and *HER-2/neu* expression". **ONCOLOGY REPORTS** 11(6): 1241-1252, 2004. Regular Article.

13. **Javier A. Menendez**, Bharvi P. Oza, Ella Atlas, Vishal Verma, Inderjit Mehmi, and Ruth Lupu. "Inhibition of tumor-associated Fatty Acid Synthase activity antagonizes estradiol- and tamoxifen-induced agonist transactivation of estrogen receptor (ER) in human endometrial adenocarcinoma cells". **ONCOGENE** 23 (28): 4945-4958, 2004. Short communication.

14. **Javier A. Menéndez**, Santiago Ropero, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Overexpression and Hyperactivity of Breast cancer associated-Fatty Acid Synthase (Oncogenic antigen-519) is insensitive to normal arachidonic fatty acid-induced supression in lipogenic tissues but it is selectively inhibited by tumoricidal α-linolenic and γ-linolenic fatty acids: A novel

mechanism by which dietary fatty acids can alter mammary tumorigenesis" **INTERNATIONAL JOURNAL of ONCOLOGY** 24(6): 1369-1384, 2004. Regular Article.

15. **Javier A. Menendez**, Luciano Vellon, Inderjit Mehmi, Bharvi P. Oza, Santiago Ropero, Ramon Colomer and Ruth Lupu. "Inhibition of Fatty Acid Synthase suppresses *HER-2/neu* (c-*erb*B-2) oncogene overexpression in cancer cells". **PROCEEDINGS of the NATIONAL ACADEMY of SCIENCES USA** (Medical Sciences) 101 (29): 10715-10720. Regular Article.

16. **Javier A. Menéndez**, Ramon Colomer, and Ruth Lupu. "Inhibition of Tumor-associated Fatty Acid Synthase Activity Enhances Vinorelbine (Navelbine®)-induced Cytotoxicity and Apoptosis against Human Breast Cancer Cells". **ONCOLOGY REPORTS** 12 (2): 411-422, 2004. Regular Article.

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18. **Javier A. Menendez**, Ruth Lupu, and Ramon Colomer. "Exogenous Supplementation with Docosahexaenoic Acid (DHA; 22:6n-3) Synergistically Enhances Taxanes Cytotoxicity and Down-regulates HER-2/*neu* oncogene expression in Human Breast Cancer Cells". **EUROPEAN JOURNAL of CANCER PREVENTION** (In press) 2005. Short communication.

19. **Javier A. Menendez**, Ruth Lupu, and Ramon Colomer. "Dietary fatty acids regulate the activation status of Her-2/*neu* (c-*erb*B-2) oncogene in breast cancer cells" **ANNALS of ONCOLOGY** 15(11): 1719-1721, 2004. Letter to the editor.

20. **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "Inhibition of Fatty Acid Synthase (FAS)-dependent neoplastic lipogenesis as the mechanism of gamma linolenic acid-induced toxicity to tumor cells: An extension to Nwankwo's hypothesis". **MEDICAL HYPOTHESES** 64(2): 337-341, 2005. Regular Article.

21. **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "Why does Fatty Acid Synthase (Oncogenic antigen-519) ignore dietary fatty acids?" **MEDICAL HYPOTHESES** 64(2): 342-349, 2005. Regular Article.

22. **Javier A. Menendez**, Inderjit Mehmi, Vishal A. Verma, Poh K. Teng, and Ruth Lupu. Pharmacological inhibition of Fatty Acid Synthase (FAS): A novel therapeutic approach for breast cancer chemoprevention through its ability to suppress Her-2/*neu* (*erb*B-2) oncogene-induced malignant transformation". **MOLECULAR CARCINOGENESIS** 41(3): 164-178, 2004. Brief communication.

23. **Javier A. Menendez** and Ruth Lupu. "Fatty Acid Synthase (FAS)-catalyzed *de novo* fatty acid biosynthesis: From anabolic-energy-storage pathway in normal tissues to jack-of-all-trades in cancer cells". **ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS** 52(6): 414-426, 2004. Review.

24. **Javier A. Menendez**, Josiah P. Decker, and Ruth Lupu. "In support of Fatty Acid Synthase (FAS) as a metabolic oncogene: Extracellular acidosis acts in an epigenetic fashion up-regulating FAS gene expression in cancer cells". **JOURNAL of CELLULAR BIOCHEMISTRY** 94(1): 1-4, 2005. View point.

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26. **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "Pharmacological and Small Interference RNA-mediated inhibition of breast cancer-associated fatty acid synthase (Oncoantigen-519) synergistically enhances Taxol™ (Paclitaxel)-induced cytotoxicity". **INTERNATIONAL JOURNAL of CANCER** 2005 Jan 16 [Epub ahead of print] Fast track.

27. **Javier A. Menendez**, Ramon Colomer and Ruth Lupu. "Obesity, Fatty Acid Synthase and Cancer: Serendipity or forgotten causal linkage? **MOLECULAR GENETICS and METABOLISM** (In press; available online 9 December 2004). Letters to the editor.

28. **Javier A. Menendez** and Ruth Lupu. "RNA-interference-mediated silencing of the p53 tumor-suppresor protein (TP53) drastically increases apoptosis after inhibition of endogenous fatty acid metabolism in breast cancer cells: Association of low levels of TP53 with breast cancer sensitivity to Fatty Acid Synthase inhibition". **INTERNATIONAL JOURNAL of MOLECULAR MEDICINE** 15 (1): 33-40, 2005. Regular Article.

29. **Javier Abel Menendez**, Luciano Vellon, Ramon Colomer, and Ruth Lupu. "Oleic Acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* (*erb*B-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin™) in breast cancer cells with Her-2/*neu* oncogene amplification". **ANNALS of ONCOLOGY** 2005 Jan 10 [Epub ahead of print]. Article.

30. **Javier A. Menendez**, Luciano Vellon, Bharvi P. Oza, and Ruth Lupu. "Does endogenous fatty acid metabolism allow cancer cells to sense hypoxia and mediate hypoxic vasodilatation? Characterization of a novel molecular connection between Fatty Acid Synthase (FAS) and Hypoxia-inducible factor-1α (HIF-1α)-related expression of Vascular Endothelial Growth Factor (VEGF) in cancer cells overexpressing Her-2/*neu* oncogene. **JOURNAL of CELLULAR BIOCHEMISTRY** 2005 Jan 24 [Epub ahead of print]. View point.

31. Luciano Vellon, **Javier A. Menendez**, David G. Griggs, and Ruth Lupu. "$\alpha_v\beta_3$ Integrin Regulates Heregulin (HRG)-induced cell proliferation and survival in breast cancer". **ONCOGENE** (In press, 2005). Article.

32. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "The anti-obesity drug Orlistat induces cytotoxic effects, suppresses Her-2/*neu* (*erb*B-2) oncogene overexpression, and synergistically interacts with trastuzumab (Herceptin) in chemoresistant ovarian cancer cells". **INTERNATIONAL JOURNAL of GYNECOLOGICAL CANCER** (In press, 2005) Short report.

33. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "The statin Orlistat: From anti-obesity drug to anti-cancer agent in Her-2/*neu* (*erb*B-2)-overexpressing gastrointestinal tumors? **EXPERIMENTAL BIOLOGY and MEDICINE** March 1, 2005; 230 (3). Comment.

34. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "Targeting Fatty Acid Synthase (FAS)-driven lipid rafts: A novel strategy to overcome trastuzumab resistance in breast cancer cells" **MEDICAL HYPOTHESES** (In press, available online 24 December 2004). Article.

35. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin-triggered Her-2/*neu* signaling enhances nuclear accumulation of $p21^{WAF1/CIP1}$ and protects breast cancer cells from cisplatin-induced genotoxic damage" **INTERNATIONAL JOURNAL of ONCOLOGY** 26(3): 649-660, 2005.

36. **Javier A. Menendez**, Bharvi P. Oza, Ramon Colomer, and Ruth Lupu. "The estrogenic activity of synthetic progestins used in oral contraceptives enhances Fatty Acid Synthase (FAS)-dependent breast cancer cell proliferation and survival" **INTERNATIONAL JOURNAL of ONCOLOGY** (In press, 2005). Article.

37. Alejandro Vazquez-Martin, Santiago Ropero, **Javier A. Menendez**, and Ramon Colomer. "Growth and molecular interactions between Tamoxifen and Trastuzumab" Re. to: Argiris A. et al. "Synergistic interactions between tamoxifen and trastuzumab (Herceptin)". **CLINICAL CANCER RESEARCH**. (In press, 2005) Letter to the editor.

38. **Javier A. Menendez**, Luciano Vellon, Bharvi P. Oza, and Ruth Lupu. "The angiogenic inducer CYR61 synergistically enhances Heregulin-stimulated secretion of Vascular Endothelial Growth Factor (VEGF) via $\alpha_v\beta_3$ integrin receptor: A novel therapeutic target for small peptidomimetic $\alpha_v\beta_3$ integrin antagonists". **INTERNATIONAL JOURNAL of MOLECULAR MEDICINE** (In press, 2005). Article.

39. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "DNA Topoisomerase IIα (TOP2A) inhibitors up-regulate Fatty Acid Synthase (FAS) gene expression in SK-Br3 breast cancer cells: *In vitro* evidence for a "functional amplicon" involving FAS, Her-2/neu and TOP2A genes". **INTERNATIONAL JOURNAL of MOLECULAR MEDICINE** (In press, 2005). Article.

40. **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "Development of an easy, rapad and objective method to identify Fatty Acid Synthase (Oncogenic antigen-519) inhibitors with potential anticancer value". **ONCOLOGY REPORTS** (In press, 2005). Article.

41. **Javier A. Menendez**, Ruth Lupu, and Ramon Colomer. "Targeting Fatty Acid Synthase (FAS): Potential for therapeutic intervention in Her-2/neu-overexpressing breast cancer" **DRUG NEWS and PERSPECTIVES** (In press, 2005). Review.

42. Ramon Colomer and **Javier A. Menendez**. "El ácido oleico inhibe el oncogén Her-2/*neu* (*erb*B-2) en el cáncer de mama" **MERCACEI** (In press, 2005). Review. (Spanish).

B. Submitted (Manuscripts under consideration)

1. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "Anti-tumoral actions of the anti-obesity drug Orlistat (Xenical™) in breast cancer cells: Blockade of cell cycle progression, promotion of apoptotic cell death and PEA3-mediated transcriptional repression of Her-2/*neu* (*erb*B-2) oncogene". **ANNALS of ONCOLOGY**. 2005. Article.

2. **Javier Abel Menendez**, Luciano Vellon, Ramon Colomer, and Ruth Lupu. "The herbal dietary supplement Gamma-Linolenic Acid (GLA; 18:3n-6) represses the transcriptional activity of Her-2/*neu* (*erb*B-2) oncogene *via* the *Ets* protein Polyomavirus Enhancer Activator 3 (PEA3)" 2004. **JOURNAL of the NATIONAL CANCER INSTITUTE**. 2005. Brief communication.

3. **Javier A. Menendez**, Bharvi P. Oza, Ramon Colomer, and Ruth Lupu. "Breast Cancer Endogenous Fatty Acid Metabolism regulates the transcriptional activity

of Estrogen Receptor-α". **PROCEEDINGS of the NATIONAL ACADEMY of SCIENCES USA** (2005). Article.

4. **Javier A. Menendez**, Bharvi P. Oza, and Ruth Lupu "Fatty Acid Synthase (FAS) gene expression and FAS-catalyzed *de novo* fatty acid biosynthesis is necessary for estrogen-stimulated breast cancer cell proliferation and survival. **JOURNAL of ENDOCRINOLOGY** (2005). Article.

C. Manuscripts in preparation

1. **Javier A. Menendez** and Ruth Lupu. "Inhibition of Fatty Acid Synthase (FAS) activity reverses tamoxifen resistance in Her-2/*neu*-overexpressing breast cancer cells" 2005. Article.

2. **Javier A. Menendez** and Ruth Lupu. "Heregulin induces transcriptional activation of breast cancer-associated Fatty Acid Synthase (Oncogenic antigen-519) by an autocrine mechanism that requires activation of Her-2/*neu*, mitogen-activated protein kinase and phosphatidylinositol 3'-kinase" 2005. Article.

3. **Javier A. Menendez**, Susan K. Peirce, Laila Siddiqui, Wen Y. Chen and Ruth Lupu. "Characterization of a novel cross-talk involving Prolactin, Prolactin Receptor, Progesterone Receptor and Fatty Acid Synthase in breast cancer cells". 2005. Article.

4. **Javier A. Menendez**, Josiah P. Decker, and Ruth Lupu. "Synergistic enhancement of breast cancer-associated Fatty Acid Synthase (FAS) expression by Peroxisome Proliferator-Activated Receptor γ (PPARγ) Ligands and Estrogen Receptor (ER) Agonists *via* Sterol-responsive elements in the FAS gene promoter" 2005. Article.

5. **Javier A. Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin overexpression protects human breast cancer cells from Cisplatin-induced Cell Death: Synergistic sensitization by trastuzumab in the absence of *HER-2/neu* overexpression". 2005. Article.

6. **Javier A. Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Fatty Acid Synthase is necessary for Heregulin-induced malignant transformation of human breast cancer cells". 2005. Article.

7. **Javier A. Menendez**, Bharvi P. Oza, Inderjit Mehmi, and Ruth Lupu "Evidence for a Complex Molecular Connection between Progesterone Receptor (PR) and

Fatty Acid Synthase (FAS) through Estrogen Receptor (ER) In T47-D Human Breast Cancer Cells". 2005. Article.

8. **Javier A. Menendez**, Inderjit Mehmi, Bharvi P. Oza, and Ruth Lupu. "The angiogenic inducer CCN1 (CYR61) up-regulates tumor-associated Fatty Acid Synthase (FAS) gene expression in breast cancer cells *via* MAPK and PI-3'K/AKT signaling cascades". 2005. Article.

9. **Javier A. Menendez**, Inderjit Mehmi, Bharvi P. Oza, Ella Atlas, and Ruth Lupu. "Down-regulation of Heregulin β-2 Expression through Anti-sense cDNA Strategy Modulates the Sensitivity of Breast Cancer Cells to Chemotherapy". 2005. Article.

10. **Javier Abel Menendez**, Santiago Ropero, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Synergistic Cytotoxicity of Omega-3 Polyunsaturated Fatty Acids (ω-3 PUFAs) and Vinorelbine in Human Breast Cancer Cells: Relationship to ω-3 PUFAs Oxidative Status". 2005. Article.

11. **Javier Abel Menéndez**, Santiago Ropero, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Omega-3 Alpha-linolenic acid Synergistically Enhances Taxanes Cytotoxicity in Human Breast Cancer Cells: Relationship to ω-3 PUFAs Oxidative Status". 2005. Article.

E. Commentaries

1. Joyce A. Nettleton. "Gamma-Linolenic acid exerts anti-estrogenic effects in hormone sensitive breast cancer cells" **PUFA NEWSLETTER** 8 (2): 18, 2004. Commentary to: **Javier A. Menendez**, Ramon Colomer, and Ruth Lupu. "ω-6 polyunsaturated fatty acid gamma-linolenic Acid (GLA; 18:3n-6) is a Selective Estrogen Response Modulator in human breast cancer cells: GLA antagonizes Estrogen Receptor (ER)-dependent transcriptional activity, transcriptionally represses ER expression, and synergistically enhances Tamoxifen and ICI 182,780 (Faslodex) efficacy in human breast cancer cells". ***International Journal of Cancer*** 109(6): 949-954, 2004.

2. "New Evidence of Protein's Role in Cancer Development" **National Cancer Institute Cancer Bulleting** 1(31): 4-5, 2004. Commentary to: **Javier A. Menendez**, Luciano Vellon, Inderjit Mehmi, Bharvi P. Oza, Santiago Ropero, Ramon Colomer and Ruth Lupu. "Inhibition of Fatty Acid Synthase suppresses *HER-2/neu* (c-*erb*B-2) oncogene overexpression in cancer cells". ***Proceedings of the National Academy of Sciences USA*** (Medical Sciences) 101 (29): 10715-10720.

3. "Research Highlights (Gene Expression): Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells" **Nature Reviews Cancer** 4: 573 (2004). Commentary to: **Javier A. Menendez**, Luciano

Vellon, Inderjit Mehmi, Bharvi P. Oza, Santiago Ropero, Ramon Colomer and Ruth Lupu. "Inhibition of Fatty Acid Synthase suppresses *HER-2/neu* (c-*erb*B-2) oncogene overexpression in cancer cells". ***Proceedings of the National Academy of Sciences USA*** (Medical Sciences) 101 (29): 10715-10720.

4. Roxanne Nelson. "Oleic acid suppresses overexpression of ERBB2 oncogene" **Lancet Oncology** 6(2), 2005. Commentary to: **Javier Abel Menendez**, Luciano Vellon, Ramon Colomer, and Ruth Lupu. "Oleic Acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* (*erb*B-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin™) in breast cancer cells with Her-2/*neu* oncogene amplification". ***Annals of Oncology*** 2005 Jan 10 [Epub ahead of print].

F. Abstracts

1. **J. A. Menéndez**, M. M. Barbacid, S. Montero, H. Cortés-Funes and Ramón Colomer. "Effect of Oleic acid on the Chemosensitivity of Breast Cancer Cells" **European Journal of Cancer**, 1999; Vol. 35, Supplement 2, p. S14 April. Abstract.
VIII Congress Spanish Association for Cancer Research (ASEICA)
April 19-23, 1999, Sitges, Barcelona, Spain.

2. M. M. Barbacid, S. Montero, **J. A. Menéndez**, H. Cortés-Funes and R. Colomer. "Kinetic analysis of a Combination of Gemcitabine with Vinorelbine in Human Breast Adenocarcinoma MCF-7 cells *in vitro*". European Journal of Cancer, 1999; Vol. 35, Supplement 2, p. S14 April. Abstract
VIII Congress Spanish Association for Cancer Research (ASEICA)
April 19-23, 1999, Sitges, Barcelona, Spain.

3. **J. A. Menéndez**, M. M. Barbacid, S. Montero, E. Escrich, H. Cortés-Funes and R. Colomer. "Taxol and Vinorelbine Cytotoxicity in Human Breast Cancer Cell Lines is Enhanced by Oleic Acid" Proceedings III International Symposium Changes in the Treatment of Breast Cancer, p. 137, June 2-4.
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June 2-4, 1999, Madrid, Spain.

4. **Menendez J. A.**, del Mar Barbacid M., Montero, S., et al. "Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells"
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5. **Menéndez, J. A.**, Ropero, S., Montero, S., Barbacid, M. M., Funes, H-C., and Colomer, R. "Oncogenic Antigen 519 (OA-519) Activity and Expression are

Regulated by Fatty Acids in Human Breast Cancer Cells" Proc. Am. Assoc. Cancer Res. Abstract # 1681.
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March 24-28, 2001, New Orleans, USA.

6. Montero, S., **Menéndez, J. A.**, Ropero, S., Barbacid, M. M., Funes, H-C., and Colomer, R. "Angiogenin Expression is Regulated by Steroid Hormones in Breast Cancer Cells" Proc. Am. Assoc. Cancer Res. Abstract #1275.
American Association for Cancer Research 92nd Annual Meeting
March 24-28, 2001, New Orleans, USA.

7. R. Colomer, S. Montero, S. Ropero, and **J. A. Menéndez** "Proyectos de Investigación Actuales en el Hospital 12 de Octubre" Encuentros en Oncología, p. 129-132.
Encuentros en Oncología
April 5-6, 2001 Segovia, Spain.

8. S. Ropero, **J. A. Menéndez**, S. Montero, H-C. Funes, and R. Colomer. "Interactions of Herceptin and Tamoxifen in HER2+ER+ Human Breast Cancer Cells"
IV Madrid Breast Cancer Conference: Changes in the Treatment of Breast Cancer
June 7-9, 2001, Madrid, Spain.

9. **J. A. Menéndez**, S. Ropero, S. Montero, H-C. Funes, and R. Colomer. "Cerulenin –a Potent Inhibitor of Fatty Acid Synthase- Produces Cytotoxicity Through the p53/p21$^{WAF1/CIP1}$ Pathway and Synergizes with Anti-tubule Agents in Breast Cancer Cells"
IV Madrid Breast Cancer Conference: Changes in the Treatment of Breast Cancer
June 7-9, 2001, Madrid, Spain.

10. **Javier Abel Menendez**, Santiago Ropero, Alejandro Vazquez-Martin, Hernan Cortes-Funes, Ruth Lupu, and Ramon Colomer "Trastuzumab and Cerulenin Synergistically Inhibit Breast Cancer Cell Growth: Interaction of HER-2/neu and Fatty Acid Synthase Signaling Pathways" Proc. Am. Assoc. Cancer Res. Abstract #2989.
American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

11. Alejandro Vazquez-Martin, **Javier Abel Menendez**, Santiago Ropero, Sagrario Montero, Hernan Cortes-Funes, and Ramon Colomer "Anthracycline Resistance on Breast Cancer Cells is Reversed by Inhibiton of Fatty Acid Synthase" Proc. Am. Assoc. Cancer Res. Abstract #4709.

American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

12. Santiago Ropero, **Javier Abel Menendez**, Sagrario Montero, Alejandro Vazquez, Hernan Cortes-Funes, and Ramon Colomer "Tamoxifen-induced Up-Regulation of HER2 Impairs the Sensitivity to Herceptin in ER+HER2+ Breast Carcinoma Cells" Proc. Am. Assoc. Cancer Res. Abstract #4978.
American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

13. **Javier A. Menendez**, I. Mehmi, E. Atlas, M-S. Tsai, and R. Lupu. "Role of Heregulin in Breast Cancer Angiogenesis"
3rd Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program
September 25-28, 2002, Orlando (Florida), USA.

14. **Javier Abel Menendez**, Ramon Colomer, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin/HER2 Signaling Up-Regulates Fatty Acid Synthase (Oncoantigen-519) Expression in Human Breast Cancer Cells Through Activation of the Phosphatidylinositol 3'-Kinase/AKT Kinase Pathway" Abstract #22.
ENH Research Reception
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15. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and Ruth Lupu. "The Angiogenic Factor CYR61, a Downstream Effector of Heregulin, Protects Breast Cancer Cells from Paclitaxel-induced Cell Death Through Integrin $\alpha_v\beta_3$" Abstract #366. **European Journal of Cancer** Vol. 38, Supplement 7, p. 108. November 2002.
14th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
November 19-22, 2002, Frankfurt, Germany.

16. Ruth Lupu, and **Javier A. Menendez**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway". **Tumor Progression Control and Hormones (non-steroidal) Session** of the **International Congress on Hormonal Steroids and Hormones and Cancer.**
Fukuoka, Japan, October 21-25, 2002.

17. **Javier A. Menendez**, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, David Griggs, and Ruth Lupu. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the

$\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway" **International Journal of Molecular Medicine.** November 2002.
7th International Meeting on Molecular Oncology
Crete, Greece, 2002.

18. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "A Molecular Cross-communication Between Heregulin/HER2 and Fatty Acid Synthase in Breast Cancer Cells". Proc. Am. Assoc. Cancer Res. Abstract #6207
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

19. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin Overexpression Protects Human Breast Cancer Cells from Cisplatin-induced Cell Death: Synergistic Sensitization by Herceptin". Proc. Am. Assoc. Cancer Res. Abstract #4053
Selected for presentation in the **Experimental/Molecular Therapeutics Poster Discussion Session** of the **American Association for Cancer Research 94th Annual Meeting**
April 5-9, 2003. Toronto, Canada.

20. Alejandro Vazquez, Santiago Ropero, **Javier Abel Menendez**, Montserrat Solanas, Eduard Escrich, Ramon Colomer. "Fatty Acid Synthase Regulates Estrogen Receptor in Breast Carcinoma Cells: Relationship to $p21^{WAF-1/CIP-1}$"
Proc. Am. Assoc. Cancer Res. Abstract #2788
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

21. Ella Atlas, Inderjit Mehmi, **Javier A. Menendez**, Hengameh Zahedkargaran, and Ruth Lupu. "The Immunoglobulin-like Domain of HRGβ-2 is Sufficient for the Chemosensitization and Inhibition of E_2-dependent Colony Formation in MCF-7 cells".
Proc. Am. Assoc. Cancer Res. Abstract #5949
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

22. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin Overexpression Protects Breast Cancer Cells From Cisplatin-induced Cell Death: Involvement of $p21^{WAF1/CIP1}$ and Synergistic Sensitization by Herceptin in the absence of *HER-2/neu* Overexpression." Abstract #12.
ENH Research Reception
March 20, 2003, Evanston (Illinois), USA.

23. Ruth Lupu, Inderjit Mehmi, Ella Atlas, and **Javier Abel Menendez**. "Overexpression of Heregulin Protects Breast Cancer Cells From Cisplatin-

induced Cell Death: Synergistic Sensitization by Trastuzumab in the Absence of *HER-2/neu* Overexpression"
44th Annual Clinical Conference Molecular Therapeutics for Cancer Metastasis. Abstract #19.
March 18-21, 2003. Houston, Texas.

24. **Javier Abel Menendez**, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Therapeutic Implications of a Novel Bidirectional Molecular Cross-talk Between the *HER-2/neu* and Fatty Acid Synthase Signaling Pathways in Breast Cancer"
44th Annual Clinical Conference Molecular Therapeutics for Cancer Metastasis. Abstract #20.
March 18-21, 2003. Houston, Texas.

25. **Javier A. Menendez**, Inderjit Mehmi, David Griggs, and Ruth Lupu. "The angiogenic factor CCN1 (CYR61) protects breast cancer cells from Taxol-induced cell death"
5th Annual Lynn Sage Breast Cancer Symposium Agenda.
September 17-21. Chicago, Illinois.

26. **J. A. Menendez**, I. Mehmi, E. Atlas, and R. Lupu. "Overexpression of Heregulin protects breast cancer cells from cisplatin-induced cell death: Synergistic sensitization by trastuzumab in the absence of *HER-2/neu* overexpression"
8th World Congress on Advances in Oncology and 6th International Symposium on Molecular Medicine. October 16-18, 2003. Crete, Greece.

27. **Javier Abel Menendez**, Inderjit Mehmi, Bharvi P. Oza, Vishal A. Verma, and Ruth Lupu. "The fatty acid synthase inhibitor C75 is a novel selective estrogen response modulator (SERM) in human endometrial adenocarcinoma cells" Abstract #11.
ENH Research Institute and Faculty Practice Associates Poster Reception
October 16, 2003, Burch Community Center, Evanston (Illinois), USA.

28. **Javier A. Menendez**, Bharvi P. Oza, Ella Atlas, Inderjit Mehmi, and Ruth Lupu "Fatty acid synthase (FAS) inhibitor C75 induces hyperactivation of ERK1/2 MAPK-Estrogen receptor alpha (ER-alpha) cross-talk, loss of ER-alpha expression, inhibition of cell growth, and apoptotic cell death in hormone-dependent breast cancer cells"
2003 AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics Abstract # A157
November 17-21, 2003, Boston (MA), USA.
Published as a Supplement to **Clinical Cancer Research**,
Volume 9, Issue 16 (December 1, 2003).

29. Ruth Lupu, Inderjit Mehmi, Bharvi P. Oza, Vishal A. Verma, Ella Atlas, and **Javier A. Menendez**. "Pharmacological blockade of tumor-associated Fatty Acid Synthase (FAS) activity antagonizes estradiol- and tamoxifen-induced agonist transactivation of estrogen receptor-alpha (ER-alpha) in human endometrial adenocarcinoma cells"
2003 AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics Abstract # A158
November 17-21, 2003, Boston (MA), USA.
Published as a Supplement to **Clinical Cancer Research**,
Volume 9, Issue 16 (December 1, 2003).

30. Manish Patel, **Javier Menendez**, Susan Peirce, and Ruth Lupu. "Fatty Acid Synthase (FAS) expression is linked to heregulin overexpression *in vivo*"
Proc. Am. Assoc. Cancer Res. Abstract # 451.
American Association for Cancer Research 95th Annual Meeting
March 27-31, 2004, Orlando (FL), USA.

31. Luciano Vellon, **Javier Abel Menendez**, and Ruth Lupu. "Heregulin regulates alpha$_v$ beta$_3$ ($\alpha_v\beta_3$) integrin expression in breast cancer cells"
Proc. Am. Assoc. Cancer Res. Abstract # LB-77.
American Association for Cancer Research 95th Annual Meeting
March 27-31, 2004, Orlando (FL), USA.

32. **Javier A. Menendez**, Bharvi P. Oza, Ella Atlas, Alejandro Vazquez-Martin, Ramon Colomer, and Ruth Lupu. "The anorectic Fatty Acid Synthase (FAS) inhibitor C75 regulates genomic and non-genomic estrogen receptor-alpha activities in breast cancer cells"
Proc. Am. Assoc. Cancer Res. Abstract # LB-268.
American Association for Cancer Research 95th Annual Meeting
March 27-31, 2004, Orlando (FL), USA.

33. **Javier A. Menendez**, Luciano Vellon, Ramon Colomer, and Ruth Lupu. "ω-9 oleic acid, the main monounsaturated fatty acid of olive oil, suppresses *HER-2/neu* (*erb*B-2) expression and synergistically enhances trastuzumab (Herceptin) efficacy in breast cancer cells with *HER-2/neu* oncogene amplification"
Proc. Am. Assoc. Cancer Res. Abstract # LB-317.
American Association for Cancer Research 95th Annual Meeting
March 27-31, 2004, Orlando (FL), USA.

34. **J. A. Menendez**, R. Colomer, Hernan Cortes-Funes, and Ruth Lupu. "Tumor-associated Fatty Acid Synthase hyperactivity regulates breast cancer cell sensitivity to paclitaxel (Taxol)-induced apoptosis"
Proc. Am. Assoc. Cancer Res. Abstract # LB-283.
American Association for Cancer Research 95th Annual Meeting

March 27-31, 2004, Orlando (FL), USA.

35. Ruth Lupu and **Javier A. Menendez**. "Fatty Acid Synthase (FAS) is a novel regulator of the oncogene Her-2/*neu* (c-*erb*B-2) in breast carcinomas: Molecular implications and targeted therapies"
The 3rd International Conference on Tumor Microenvironment: Progression, Therapy and Prevention
October 12-16, 2004, Prague, Czech Republic.

36. Alejandro Vazquez-Martin, Gemma Moreno, **Javier Abel Menendez**, Santiago Ropero, Cristina Oliveras, Jose Palacios, and Ramon Colomer. "Microarray gene expression analysis of fatty acid synthase (FAS) on breast cancer cell lines"
XIVth EuroCellPath Course: The impact of Genomics and Proteomics in Pathology
Girona, Spain, May 9-12, 2004.

37. A. Vazquez-Martin, G. Moreno, R. Colomer, **J. A. Menendez**, S. Ropero, C. Oliveras, J. Palacios. "Microarray gene expression analysis of fatty acid synthase (FAS) in breast cancer cell lines"
40th American Society of Clinical Oncology (ASCO) Annual Meeting
Abstract # 9690
June 5-8, 2004, New Orleans, LA, USA.

38. Ruth Lupu, Luciano Vellon, and **Javier A. Menendez**. "A novel CYR61-triggered CYR61-$\alpha_v\beta_3$ integrin loop regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway"
3rd International Workshop on the CCN family of genes
October 20-23, 2004, Saint-Malo, France.

39. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "The anti-obesity drug Orlistat blocks cell cycle progression and synergistically enhances trastuzumab-induced apoptotic cell death by promoting PEA3-mediated transcriptional repression of Her-2/*neu* (*erb*B-2) oncogene in cancer cells"
6th Lynn Sage Breast Cancer Symposium
Abstract #B15
October 30, 2004, Chicago, IL, USA.

40. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "Inhibition of breast cancer-associated Fatty Acid Synthase (FAS) synergistically enhances Taxol™-induced apoptotic cell death"
6th Lynn Sage Breast Cancer Symposium
Abstract #B16
October 30, 2004, Chicago, IL, USA.

41. **Javier A. Menendez**, Luciano Vellon, and Ruth Lupu. "Characterization of a CYR61-triggered "CYR61-$\alpha_v\beta_3$ autocrine loop" in the epithelial compartment of breast carcinoma that regulates cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway"
6th Lynn Sage Breast Cancer Symposium
Abstract #B17
October 30, 2004, Chicago, IL, USA.

42. Luciano Vellon, **Javier A. Menendez**, and Ruth Lupu. "$\alpha_v\beta_3$ regulates Heregulin (HRG)-induced cell proliferation and survival in breast cancer"
6th Lynn Sage Breast Cancer Symposium
Abstract #B18
October 30, 2004, Chicago, IL, USA.

43. Luciano Vellon, **Javier A. Menendez**, and Ruth Lupu. "$\alpha_v\beta_3$ integrin is a novel molecular marker of breast cancer chemosensitivity"
6th Lynn Sage Breast Cancer Symposium
Abstract #B19
October 30, 2004, Chicago, IL, USA.

44. **Javier A. Menendez**, Luciano Vellon, Ramon Colomer and Ruth Lupu. "Pharmacological and small interference RNA-induced inhibition of the lipogenic enzyme fatty acid synthase synergistically enhances Taxol (Paclitaxel)-induced apoptotic breast cancer cell death" Abstract # 306.
Poster Discussion Session: Growth Factor Pathways and Drug Resistance
27th Annual San Antonio Breast Cancer Symposium
December 8-12, 2004, San Antonio, TX, USA.

RELEVANT RESEARCH EXPERIENCE (TECHNIQUES)

- **Basic Microbiological Techniques**: Bacterial culture and isolation. Bacteriophages culture, propagation, quantification and isolation. Isolation of viral genetic material.

- **Basic Recombinant DNA Techniques**: Culture and isolation of bacterial host. Clonation vectors (plasmids, M13 sequencing vectors and integration vectors). DNA isolation, sequencing and amplification.

- **Cell Culture of mammalian cells.**

- **Electrophoresis and Western Blotting Techniques.**

- **Zimography Technique (Metalloproteinases detection).**

- **Proliferation, Adhesion and Migration/Invasion (Boyden chambers) assays.**
- **ELISA technique.**
- **DNA and RNA isolation techniques.**
- **PCR and RT-PCR techniques.**
- **Chemosensitivity Assays (Anchorage-Dependent Cytotoxicity Assays, Anchorage Independent Soft-Agar Colony Formation Assays).**
- **Pre-Clinical Analysis of Drug Interactions: Isobologram Analysis (Berenbaum Method) and Median-Effect Plot Analysis (Chou & Talalay Method).**
- **Southern Blotting technique.**
- **Immunofluorescence Microscopy and Confocal Immunofluorescence Microscopy.**
- **Gene Retroviral Infections.**
- **Transient transfections.**
- **Terminal Restriction Fragment (TRF) Length: Telomere Length.**
- **Telomerase Activity (TRAP assays): Telomeric Repeat Amplification Protocol.**
- **Luciferase assays.**
- **Gene silencing: RNA interference techniques (siRNA).**
- **Flow cytometry, Cell Cycle.**

SCIENTIFIC MEETINGS and CONFERENCES

- ✓ "HARD DISEASES: CANCER AND AIDS" April-June, 1993
  Oviedo University, Spain.
- ✓ "A VACCINE AGAINST MALARIA: THE FUTURE OF SINTETIC VACCINES" 1995,
  Oviedo University, Spain.
- ✓ "THE BIOTECHNOLOGY AND ITS INDUSTRIAL APLICATIONS" 1995 and 1996
  Oviedo University, Spain.

- ✓ "VIII SYMPOSIUM on HUMAN FERTILITY" May 16, 1997
  Oviedo, Spain.
- ✓ "DEVELOPMENT OF NEW THERAPIES AGAINST CANCER" May 26, 1998
  José Casares Gil Foundation, Madrid, Spain.
- ✓ "10th AVANCED COURSE IN MEDICAL ONCOLOGY" June 1-4, 1998
  European School of Oncology; Madrid, Spain.
- ✓ "CELL CYCLE GENETIC ALTERATIONS IN NEOPLASTIC PROCESSES: BIOLOGICAL AND CLINICAL IMPLICATIONS" June 3, 1998
  José Casares Gil Foundation; Madrid, Spain.
- ✓ "1st INTERNATIONAL SYMPOSIUM ON LUNG CANCER: PROGRESS IN THE TREATMENT OF LUNG CANCER" June 5, 1998
  European Organization for Research and Treatment of Cancer (EORTC); Madrid, Spain.
- ✓ "VI ONCOGENES SYMPOSIUM" October 5-6, 1998
  Spanish Association for Cancer Research (ASEICA); Madrid, Spain.
- ✓ "PROTEIN AND NUCLEIC ACID CHEMISTRY" October 9, 1998
  Madrid, Spain.
- ✓ "NEW TARGETS IN THE TREATMENT OF CANCER: RATIONAL DEVELOPMENT OF ANTINEOPLASTIC DRUGS" October 13, 1998
  Madrid, Spain.
- ✓ "CANCER: BIOLOGY AND ANTI-CANCER AGENTS" October 29-30, 1998
  Spanish Association for Cancer Research (ASEICA)
  Granada, Spain.
- ✓ "MEDICAL ONCOLOGY TO PRIMARY ATTENTION" November 24-26, 1998
  Madrid, Spain.
- ✓ "II INTERNATIONAL SYMPOSIUM ON MOLECULAR DIAGNOSIS IN MEDICINE" November 26-27, 1998
  Madrid, Spain.
- ✓ "VIII CONGRESS SPANISH ASSOCIATION FOR CANCER RESEARCH (ASEICA)" and "I JOINT MEETING ASEICA-SPANISH ASSOCIATION FOR MEDICAL ONCOLOGY (SEOM)" April 19-21, 1999
  Sitges (Barcelona), Spain.
- ✓ "III INTERNATIONAL SYMPOSIUM CHANGES IN THE TREATMENT OF BREAST CANCER" June 2-4, 1999
  European Society of Medical Oncology (ESMO); Madrid, Spain.

- ✓ "VI ONCOGENES AND CANCER SYMPOSIUM" May 10-11, 2000.
  Madrid, Spain.
- ✓ "ANTI-PROLIFERATIVE EFFECT OF CANNABINOIDES" July 4, 2000.
  Madrid, Spain.
- ✓ "INHIBITORS of TRANSDUCTION SIGNALING PATHWAYS" February 8, 2001.
  Madrid, Spain.
- ✓ "92nd AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING" March 24-28, 2001
  New Orleans, USA.
- ✓ "ENCUENTROS EN ONCOLOGIA" April 5-6, 2001
  Segovia, Spain.
- ✓ "IV Madrid BREAST CANCER CONFERENCE: CHANGES IN THE TREATMENT OF BREAST CANCER" June 7-9, 2001
  Madrid, Spain.
- ✓ "Simply SERMS: A view of the chemoprevention of breast cancer"
  50th Anniversary Cancer Research Laboratory Distinguished Lecture
  University of Berkeley, California, USA. November 27, 2001.
- ✓ "Cellular and Molecular Biology & Subcellular Structure: Postdoctoral Research Day"
  Lawrence Berkeley National Laboratory.
  University of Berkeley, California, USA. December 5, 2001.
- ✓ "93th AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING"
  April 6-10, 2002
  San Francisco, California, USA.
- ✓ "ADVANCES IN TUMORAL BIOLOGY" November 6-8, 2002
  Oviedo, Spain.
- ✓ 44th ANNUAL CLINICAL CONFERENCE "MOLECULAR THERAPEUTICS FOR CANCER METASTASIS"
  The University of Texas MD Anderson Cancer Center
  Houston, Texas, USA. March 18-21, 2003.
- ✓ "94th AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING"
  April 5-9, 2003
  Toronto, Ontario, Canada.

- ✓ "95th AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING"
  March 26-31, 2004
  Orlando, Florida, USA.

- ✓ Sixth Annual LYNN SAGE BREAST CANCER SYMPOSIUM
  October 30, 2004
  Chicago, Illinois, USA

ADDITIONAL ACADEMIC INFORMATION

MASTER DEGREE PROGRAM in "BIOCHEMISTRY AND MOLECULAR BIOLOGY"
Complutense University, Madrid, Spain:

- ❖ "CELL CULTURES: TECHNIQUES AND APPLICATIONS"
  November 27, 1998-January 22, 1999
- ❖ "STRUCTURE OF PROTEINS"
  November 30, 1998-January 27, 1999
- ❖ "CELL PROLIFERATION, DIFFERENTIATION AND DEVELOPMENT"
  February 15-March 15, 1999
- ❖ "CHARACTERIZATION OF MACROMOLECULES"
  April 15-May 30, 1999
- ❖ "EXTRACELLULAR MATRIX: STRUCTURE AND FUNCTION"
  May 21-June 7, 1999
- ❖ "DINAMIC OF BIOLOGICAL MEMBRANES"
  November 24, 1999-February 24-2000
- ❖ "ONCOGENES AND CELLULAR MALIGNIZATION: TUMORAL MARKERS"
  May 10-11, 2000
- ❖ "LIPIDIC MESSAGERS IN SIGNAL TRANSDUCTION PATHWAYS"
  June 19-July 7, 2000

LANGUAGES

- SPANISH: Mother tongue.
- ENGLISH: Advanced.
- FRENCH: Advanced.