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VIEWPOINT

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

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Abstract Her-2/*neu* (*erb*B-2) oncogene overexpression is associated with increased tumor progression and metastasis. Fatty acid synthase (FAS), the key lipogenic enzyme responsible for the endogenous synthesis of fatty acids, has been shown to be one of the genes regulated by Her-2/*neu* at the level of transcription, translation, and biosynthetic activity. Interestingly, we recently established that both pharmacological inhibition of FAS activity and silencing of FAS gene expression specifically suppress Her-2/*neu* oncoprotein expression and tyrosine-kinase activity in breast and ovarian Her-2/*neu* overexpressors. Unraveling the functional organization of this novel bi-directional molecular connection between Her-2/*neu* and FAS-dependent neoplastic lipogenesis is a major challenge that the field is only beginning to take on. Considering that Her-2/*neu* overexpression correlates with increased expression of the hypoxia inducible factor-1α (HIF-1α), which, in a mitogen-activated protein kinase (MAPK)-dependent manner, plays a key role in the expression of several genes including cytokines such as vascular endothelial growth factor (VEGF), we hypothesized that FAS blockade should result in a concomitant down-regulation of VEGF. Unexpectedly, the specific inhibition of the de novo fatty acid synthesis with the small-molecule inhibitor of FAS activity C75 resulted in a dramatic dose-dependent enhancement (up to 500% increase) of VEGF secretion in Her-2/*neu*-overexpressing SK-Br3, BT-474, and SKOV3 cancer cells. Concurrently, FAS blockade drastically activated MAPK and promoted further a prominent accumulation of HIF-1α in Her-2/*neu* overexpressors. Moreover, U0126-induced inhibition of MAPK activity completely abolished C75-induced up-regulation of HIF-1α expression and VEGF secretion, whereas it did not modulate C75-induced down-regulation of Her-2/*neu* oncogene. Importantly, RNA interference (RNAi)-mediated silencing of the FAS gene recapitulated C75's effects by up-regulating VEGF secretion, MAPK activation and HIF-1α expression. Therefore, it appears that perturbation of cancer-associated endogenous fatty metabolism triggers a ''hypoxia-like'' (oxygen-independent) condition that actively rescues Her-2/*neu*-dependent MAPK → HIP-1α → VEGF cascade. It is tempting to suggest that an intact FAS-catalyzed endogenous fatty acid metabolism is a necessary metabolic adaptation to support the enhanced ability of Her-2/*neu*-overexpressing cancer cells to survive cellular hypoxia in a HIF-α-dependent manner. J. Cell. Biochem. 94: 857–863, 2005. © 2005 Wiley-Liss, Inc.

Key words: Her-2/*neu*; *erb*B-2; fatty acid synthase; angiogenesis; VEGF; hypoxia; cancer

Her-2/*neu* (*erb*B-2) gene amplification is one of the most consistent alterations found in human malignancies [Akiyama et al., 1986; Slamon et al., 1987]. Although the ultimate biological pathways activated by Her-2/*neu* are not completely characterized, the oncogenic potential of Her-2/*neu* has been consistently

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established. Her-2/*neu*-transfected cells acquire a more malignant phenotype [Hudziak et al., 1987], with stimulation of cell proliferation, invasion [Ignatoski et al., 2000], and metastasis [Spencer et al., 2000]. In addition, cancer cells bearing Her-2/*neu* gene amplification have increased signaling through the *Raf*-mitogen-activated protein kinase extracellular signal-regulated kinase (ERK1/2 MAPK) pathway [Janes et al., 1994; Tzahar and Yarden, 1998]. This aberrant signaling may increase the production of cytokines such as vascular endothelial growth factor (VEGF), thus leading to favorable growth conditions in the tumor microenvironment [Petit et al., 1997]. Her-2/*neu* amplification has also been shown to correlate with increased expression of the hypoxia inducible factor-1a (HIF-1) [Laughner et al., 2001; Bos et al., 2003], which, in a MAPK-dependent manner, plays a key role in the expression of several genes including VEGF [Berra et al., 2000; Pages et al., 2000]. This Her-2/*neu*-associated increase in VEGF, in turn, leads to increased amounts of angiogenesis and decreased tumor hypoxia [Blackwell et al., 2004].

A recent transcriptome analysis of Her-2/*neu* in breast epithelial cells revealed a molecular connection to fatty acid synthase (FAS) [Kumar-Sinha et al., 2003], the key enzyme responsible for the endogenous synthesis of long-chain fatty acids through catalyzing the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA [Wakil, 1989]. Accordingly, we recently reported a positive correlation between the amplification and/or overexpression of Her-2/*neu* oncogene and high levels of FAS in breast cancer cells [Menendez et al., 2004a]. In well-nourished adults FAS-dependent de novo fatty acid biosynthesis occurs constitutively at very low levels, since the requirements for fatty acids is sufficiently met by dietary intake. However, after numerous clinical and basic research studies, it now appears that a biologically aggressive subset of carcinomas constitutively express high levels of FAS and undergo significant endogenous fatty acid biosynthesis independently of the regulatory signals that down-regulate fatty acid synthesis in normal cells [Kuhajda, 2000a]. Fascinatingly, our latest results established that pharmacological inhibition of FAS activity dramatically reduces the expression of Her-2/*neu* oncogene in cancer cells [Menendez et al., 2004b]. The specific suppression of FAS expression by RNAi-mediated silencing of the FAS gene also resulted in the repression of Her-2/*neu* expression, thus revealing a bi-directional nature of the molecular connection between Her-2/*neu* and FAS-dependent neoplastic lipogenesis.

Considering that Her-2/*neu* plays a key role in the maintenance of VEGF-dependent angiogenesis, we hypothesized that FAS blockade should result in a concomitant down-regulation of VEGF in Her-2/*neu*-overexpressing cancer cells. Her-2/*neu*-overexpressing SK-Br3, BT-474, and SKOV3 cancer cells were incubated in the absence or presence of various concentrations of the α-methylene-γ-butyrolactone C75, a small-molecule inhibitor of FAS activity that distinctively inhibits purified mammalian FAS with characteristics of a slow-binding inhibitor [Kuhajda et al., 2000b], for 48 h. Conditioned media were then assayed for the protein concentrations of the VEGF secretory isoform, $VEGF_{165}$, by ELISA (Fig. 1). In SK-Br3 breast cancer cells, the basal level of VEGF secretion was dose-dependently increased from 12 ± 1.5 in untreated control cells to 51.6 ± 6.9 pg VEGF/μg protein in cell treated with the highest concentration of C75 (10 μg/ml; Fig. 1a). VEGF also rose progressively, from 12 ± 0.1 in untreated control cells to 83 ± 3.1 pg VEGF/μg protein, when BT-474 breast cancer cells were treated with various concentrations of C75 (Fig. 1b). C75-induced FAS activity also dramatically enhanced VEGF secretion from 5 ± 0.2 to 62 ± 1.3 pg VEGF/μg protein in SKOV3 ovarian cancer cells (Fig. 1c). Remarkably, pharmacological inhibition of FAS activity had no significant effects on the levels of VEGF secretion in MCF-7 breast cancer cells which express physiological amounts of Her-2/*neu* oncogene. Thus, the basal level of VEGF secretion in C75-treated MCF-7 cells slightly increased from 4.2 ± 0.2 to 6.9 ± 0.2 pg VEGF/ $\pm$ μg protein.

VEGF expression can be induced by exposure of tumor cells to hypoxia or growth factors and, in both cases, this expression is due in part to increased VEGF gene transcription that is mediated by hypoxia-inducible factor 1α (HIF-1α), which is a heterodimeric transcription factor. HIF-1α activity is increased by both intratumoral hypoxia and genetic alterations, including loss of function mutations in tumor suppressor genes, as well as gain of functional alterations in oncogenes that activate the

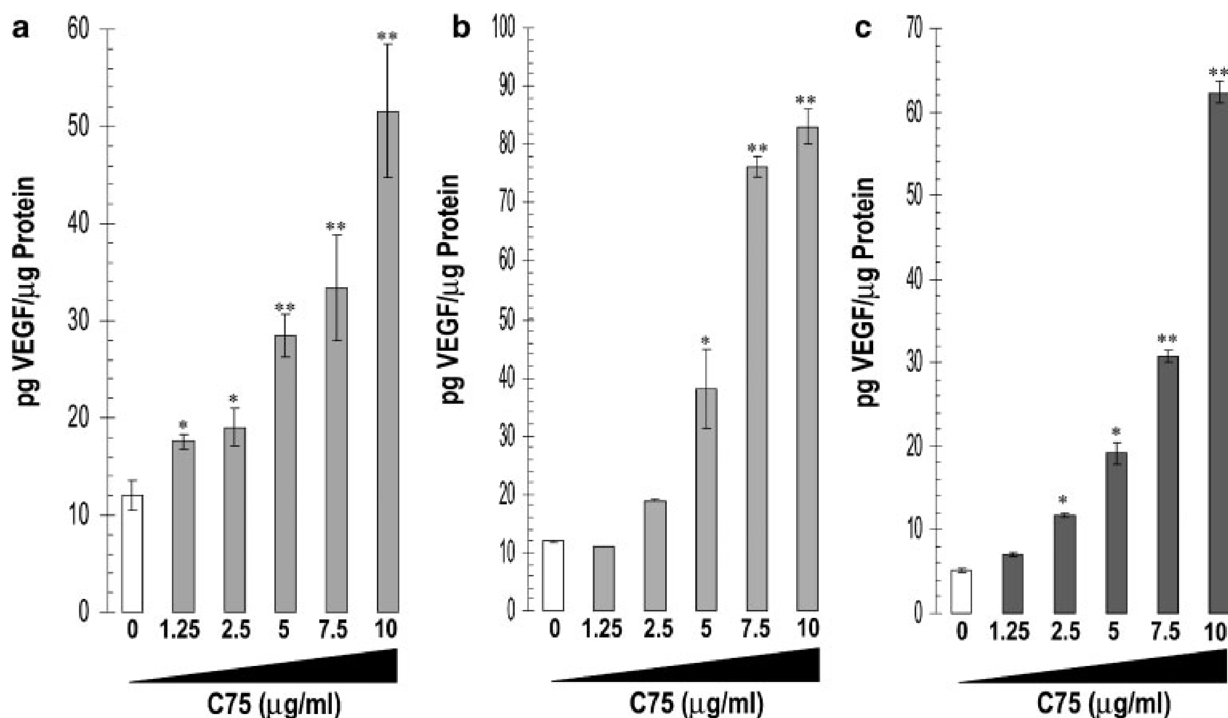

Fig. 1. **a**: Enzyme-linked immunosorbent assay analysis of secreted vascular endothelial growth factor (VEGF) protein concentrations in the conditioned media of cancer cell lines following pharmacological blockade of FAS activity. SK-Br3, BT-474, and SKOV3 cancer cell lines were seeded on 100 mm plates and cultured in complete growth medium. Upon reaching confluence, the cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and cultured in serum-free medium overnight. C75 was then added to the culture at increasing concentrations (1.25–10 μg/ml), and incubation was carried out at 37°C up to 48 h. After incubation, the conditioned medium was aspirated, centrifuged at 1,100*g* for 10 min at 4°C to remove debris and stored at −80°C until analysis. The VEGF protein level in the conditioned media was determined with the use of VEGF enzyme-linked immunosorbent assay (Human VEGF Quantikine ELISA, R&D Systems, Minneapolis, MN) as per manufacturer's instructions. Data are the mean (columns) ±SD (bars) from three independent experiments performed in duplicate. A paired Student's *t*-test was used to evaluate statistically significant differences in VEGF protein levels (in pg/mg) between the C75 treatment groups and the vehicle control group. $P < 0.05$ (*) and $P < 0.005$ (**) were selected as the statistically significant values. All statistical tests and corresponding *P* values were two-sided.

MAPK signal transduction pathway [Semenza, 2002, 2003; Powis and Kirkpatrick, 2004; Yeo et al., 2004]. Interestingly, while the content of total MAPK was similar in C75-treated and control Her-2/*neu*-overexpressing BT-474 cells, active MAPK was extensively higher in C75-treated BT-474 cells (Fig. 2a). Concurrently, FAS blockade promoted a prominent accumulation of HIF-1α protein (Fig. 2a). Importantly, pharmacological inhibition of MAPK activity abrogated C75-induced up-regulation of VEGF secretion, while C75-treated BT-474 breast cancer cells failed to accumulate HIF-1α in the presence of MAPK inhibitor U0126 (Fig. 2b). C75-induced down-regulation of Her-2/*neu*-coded $p185^{Her\text{-}2/neu}$ oncoprotein was not affected following U0126-induced inhibition of MAPK activity (Fig. 2c). Equivalent results were found in SK-Br3 and SKOV3 cancer cells (data not shown). To rule out a role for non-FAS C75-mediated effects, FAS gene expression was silenced using the potent and highly sequence-specific mechanism of RNA interference (RNAi). If C75-induced blockade of FAS activity is the most important molecular mechanism responsible for C75-induced over-secretion of VEGF, a down-regulation of FAS expression would lead to enhanced VEGF expression in MAPK-HIF-1α-related manner. Figure 2d (left panel) shows a Western blot analyses for a representative experiment ($n = 3$) in which two different concentrations of the RNAi targeted for FAS mRNA were used. At 72 h after transfection of BT-474 cells, FAS RNAi at 200 nM severely suppressed expression of FAS (up to ~90% reduction) when compared with either untransfected control cells or cells transfected with a non-specific control pool of RNAi (data not shown). Importantly, RNAi-induced FAS silencing recapitulated C75's effects by down-regulating Her-2/*neu*

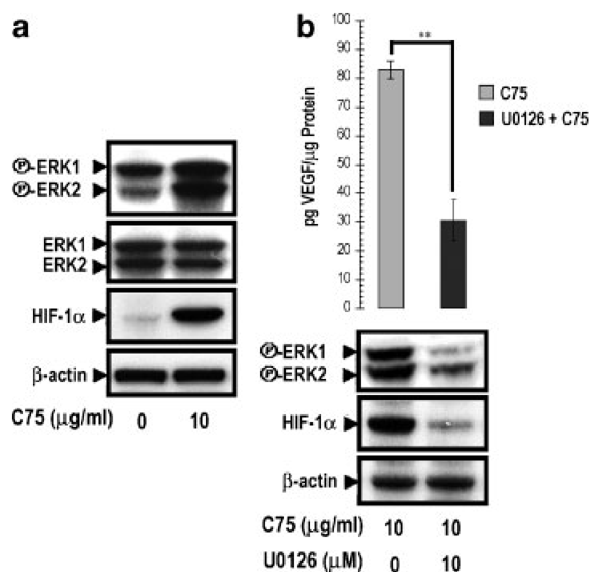

Fig. 2. **a**, **b**: Analyses of MAPK, HIF-1α, VEGF, and p185$^{Her\text{-}2/neu}$ in BT-474 breast cancer cells following pharmacological blockade of FAS activity. BT-474 cells cultured in the presence or absence of C75, U0126 or their combinations as specified for 48 h, were washed two times with cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA 150 mM, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerolphosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride) for 30 min. The lysates were cleared by centrifugation in an eppendorff tube (15 min at 14,000 rpm, 4°C). Protein content was determined against standardized controls using the Pierce Protein Assay kit (Rockford, IL). Equal amounts of protein (20 μg) were resuspended in 5× Laemli sample buffer for 10 min at 70°C, subjected to electrophoresis on 10% SDS–PAGE, and transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T (25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween 20) containing 5% (w/v) nonfat dry milk. The treated filters were washed in TBS-T and then incubated with primary antibodies overnight at 4°C with primary antibody in TBS-T containing 5% (w/v) bovine serum albumin (BSA). The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% (w/v) nonfat dry milk were added for 1 h, and primary antibody binding was detected with enhanced chemiluminescence reagent (Pierce). Blots were re-probed with an antibody for β-actin to control for protein loading and transfer. To examine the effect of FAS blockade on HIF-1α nuclear accumulation, cells were collected in cold PBS and separated into cytoplasmic and nuclear extracts. Briefly, after centrifugation, cells were resuspended in 150 μl of buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin). Cells were then incubated on ice for 10 min. The lysate was spun for 30 s to separate the nuclei and supernatant. The nuclear pellet was resuspended in 100 μl of nuclear extraction buffer (20 mM HEPES (pH 7.9), 0.45 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin) and incubated for 10 min. Thereafter, the solution was centrifugated at 18,000*g* and the supernatant (nuclear extracts) isolated. Equal amounts of protein (50 μg) were subjected to immunoblotting procedures for HIF1-α as described above. Anti-MAPK and anti-MAPK rabbit polyclonal antibodies were from Cell Signal Technology (Beverly, Maryland). Anti-HIF-1α (clone H1alpha67) mouse monoclonal antibody was from Novus Biologicals, Inc. (Littleton, CO). Anti-β-actin goat polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The VEGF protein level in the conditioned media was determined with the use of VEGF enzyme-linked immunosorbent assay as described in Figure 1. **c**: Flow cytometric analysis of cell surface-associated Her-2/*neu* oncogene expression. The specific surface expression of Her-2/*neu* was determined by flow cytometry by measuring the binding of a mouse anti-p185$^{Her\text{-}2/neu}$ antibody directed against the extracellular domain of Her-2/*neu* (clone Ab-5; Oncogene Research Products, San Diego, CA). After 48 h of treatment with C75 in the absence or presence of U0126, BT-474 cells were washed once with cold PBS and harvested by scrapping in cold PBS. The cells were pelleted and resuspended in cold PBS containing 1% FBS. The cells were then incubated with anti-p185$^{Her\text{-}2/neu}$ antibody (Ab-5) at 5 μg/ml for 1 h at 4°C. After this, the cells were washed twice with cold PBS, resuspended in cold PBS containing 1% FBS, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) diluted 1:200 in cold PBS containing 1% FBS for 45 min at 4°C. Finally, the cells were washed once in cold PBS, and flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Diego, CA) equipped with Cell Quest Software (Becton Dickinson). The mean fluorescence signal associated with cells for labeled p185$^{Her\text{-}2/neu}$ was quantified using the GEO MEAN fluorescence parameter provided with the software. Data are the mean (columns) ±SD (bars) from three independent experiments. A paired Student's *t*-test was used to evaluate statistically significant differences in p185$^{Her\text{-}2/neu}$ expression. **d**: Analyses of MAPK, HIF-1α, p185$^{Her\text{-}2/neu}$, and VEGF in BT-474 breast cancer cells following RNAi-induced silencing of *FAS* gene. The FAS-targeting siRNA duplexes were designed according to a previous report [Menendez et al., 2004b] and synthesized by Dharmacon RNA Technologies. The control siRNA duplexes containing nonspecific sequences that do not have a match in human genome were provided by Dharmacon RNA Technologies (Lafayette, CO). Transfection of BT-474 cells with the above siRNA oligonucleotides was performed with FuGENE 6 as previously described [Menendez et al., 2004b]. After 72 h of transfection in low-serum (0.1% FBS) medium, cell lysates were prepared as described above and assessed for the expression of FAS, p185$^{Her\text{-}2/neu}$, MAPK, -MAPK, HIF-1α, and β-actin (**left panels**). The primary antibody for FAS immunoblotting was a mouse IgG_1 FAS monoclonal antibody from BD Biosciences Pharmingen (clone 23, San Diego, CA). VEGF secretion in conditioned media from siRNA FAS-transfected BT-474 cell (**right panel**) was determined as described above.

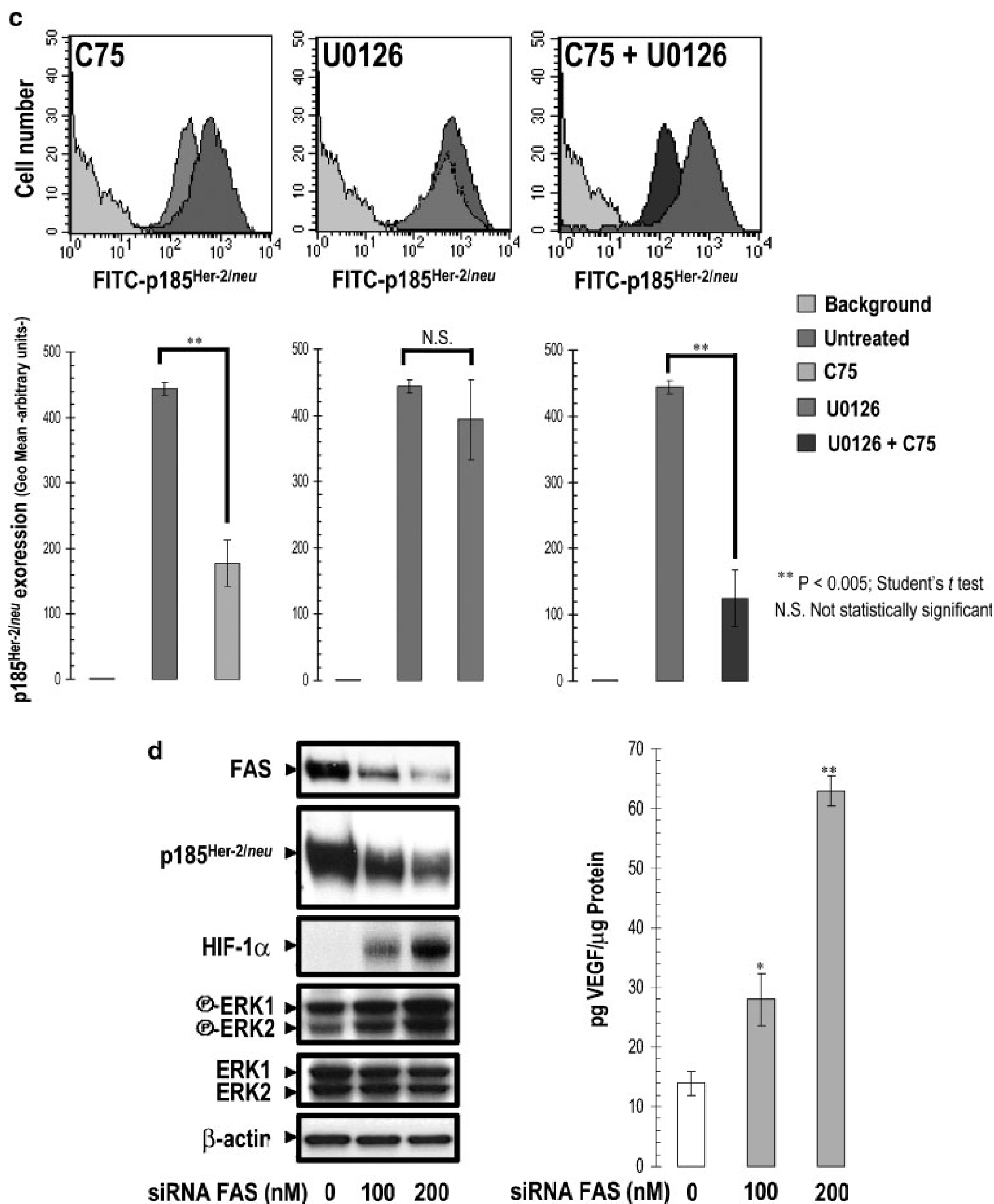

Fig. 2. (*Continued*)

expression and concomitantly promoting HIF-1α accumulation in a MAPK-related manner. Moreover, FAS RNAi-transfected BT-474 cells exhibited a dramatic increase in the basal level of VEGF secretion (Fig. 2d, right panel). These data showing that RNAi-mediated silencing of FAS gene dramatically enhances VEGF expression in a MAPK-HIF1α-related manner strongly suggest that C75 likely exerts its effects mostly through its FAS target.

These findings, altogether, strongly suggest that inhibition of cancer-associated endogenous fatty metabolism triggers a "hypoxia-like" condition that actively rescues Her-2/*neu*-dependent MAPK → HIP-1α → VEGF cascade. However, why do Her-2/*neu*-overexpressing cancer cells understand FAS blockade as an insufficiency of oxygen? We previously reported that Her-2/*neu* may act as a novel molecular sensor of energy imbalance after the perturbation of cancer-associated endogenous fatty acid metabolism [Menendez et al., 2004b]. Considering the early and almost universal activation of both FAS and Her-2/*neu* in pre-malignant lesions of malignancies such as breast cancer [Milgraum et al., 1997; DiGiovanna et al., 2002; Xu et al., 2002], it is reasonable to suggest that a tight liaison between FAS and Her-2/*neu* may occur prematurely in cancer ontogeny to facilitate a higher tolerance to limiting oxygen availability, which is known to play a key role in the growth-regulatory process underlying carcinogenesis. As proposed by Hochachka et al. [2002] and Baron et al. [2004], upon the unusual metabolic situation that takes place in the hypoxic microenvironment of pre-malignant cancer stages, the need for more oxidizing power when oxygen is restraining may be satisfied by using the FAS pathway as a means for balancing redox through its ability to consume reducing equivalents (i.e., NADPH) as part of its normal function. Moreover, it is reasonable to suggest that only the cancer cells capable of developing a FAS-dependent tolerance to limiting oxygen can survive. Concurrently, the constitutive activation of up-stream FAS regulatory cascades (i.e., MAPK) in response to early oncogenic changes including Her-2/*neu* overexpression would result in the maintenance of FAS overexpression and hyperactivity as the malignancy advances and hypoxic conditions continue to occur [Menendez et al., 2004c]. In Her-2/*neu*-promoted advanced cancer stages, the blockade of FAS-dependent endogenous fatty metabolism suppresses Her-2/*neu* expression [Menendez et al., 2004b]. Our current results reveal that a loss of this functional linkage between FAS and Her-2/*neu* obligates cancer cells to activate anti-hypoxic molecular mechanisms of cell survival including MAPK/HIF-1α-dependent up-regulation of VEGF. We recently found that acidification of the extracellular milieu acts in an epigenetic fashion activating FAS gene expression in cancer cells, whereas Her-2/*neu* overexpression significantly reduced this pH-dependent up-regulation of FAS gene expression [Menendez et al., 2004d]. Although we failed to observe an equivalent transcriptional activation of FAS gene upon hypoxic conditions, it has recently been demonstrated that a pH-dependent mechanism allows HIF-1α to activate its target genes independently of oxygen tension [Mekhail et al., 2004]. Indeed, low pH-induced activation of FAS gene expression in cancer cells is repressed in the presence of genistein, a tyrosine kinase inhibitor that inhibit HIF-1 activity by blocking the synthesis of HIF-1 subunits and/or interfering with HIF-1 DNA binding activity in hypoxia [Wang et al., 1995] (data not shown). These findings, altogether, strongly support the notion that a previously unrecognized molecular connection between FAS-catalyzed endogenous fatty acid metabolism and HIF-1α might be viewed as a novel preemptive strategy for maintaining oxygen homeostasis in Her-2/*neu*-overexpressing cancer cells. Efforts are underway to identify whether this physiological response in advanced cancer stages is determined by the past (developmental) programming of cells in pre-malignant and/or early-stages of breast and ovarian carcinomas.

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