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The Tyrosine Kinase Receptor HER2 (*erb*B-2): From Oncogenesis to Adipogenesis

Alejandro Vazquez-Martin,[1] Francisco Jose Ortega-Delgado,[2] Jose Manuel Fernandez-Real,[2] and Javier A. Menendez[1*]

[1] *Catalan Institute of Oncology (ICO), Girona Biomedical Research Institute (IdIBGi), Hospital Universitari de Girona Dr. Josep Trueta, Girona, Catalonia, Spain*

[2] *Department of Diabetes, Endocrinology and Nutrition, Girona Biomedical Research Institute (IdIBGi), CIBEROBN Obesity and Nutrition Physiopathology CB06/03/010, Girona, Catalonia, Spain*

ABSTRACT

Recent experimental evidences begin to support the notion that the *proto*-oncogene HER2 (*erb*B-2) might unexpectedly function to modulate the adipogenic conversion of preadipocytes. Two opposing scenarios have been proposed, however, to explain the influence of HER2 on adipocyte differentiation. In one hand, down-modulation of HER2 expression and pharmacological reduction of HER2 activity have been related to enhanced adipocyte differentiation. On the contrary, an increased abundance in HER2 has been described in differentiated adipocytes compared with preadipocytes. Considering that expression and activity of the lipogenic enzyme Fatty Acid Synthase (FASN) become up-regulated during adipogenic conversion, we recently hypothesized that a "HER2 → FASN axis" -a "lipogenic benefit" that has been shown to enhance cancer cell proliferation, survival, chemoresistance and metastasis in biologically aggressive subgroups of breast carcinomas-might also *naturally* work during the differentiation of preadipocytes. To definitely clarify if the discrepancy between the opposing theories for a role of HER2 during adipocyte differentiation related to the experimental approach utilized to compare the abundance of HER2 in undifferentiated and differentiated adipocytes (i.e., cell lysates containing equivalent protein content versus cell lysates generated from similar cell numbers), we here took advantage of a high content microscopy approach. Using an automated confocal imaging platform, we monitored the expression status of the adipogenic marker FASN and its timing relationship with HER2 not only in individual 3T3-L1 cells but further in whole cultures of 3T3-L1 preadipocytes undergoing adipogenic conversion. Our findings not only confirm a *non-oncogenic* role for HER2 in the process of adipose differentiation but further suggest that HER2 might represent a previously unrecognized target to manage obesity via the lipogenic enzyme FASN. J. Cell. Biochem. 105: 1147–1152, 2008.

KEY WORDS: HER2; FATTY ACID SYNTHASE; ONCOGENESIS; ADIPOGENESIS

HER2 (*ERB*B2; Her-2/*neu*) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) that regulates biological functions as diverse as cellular proliferation, transformation, differentiation, motility, and apoptosis [Rubin and Yarden, 2001; Yarden, 2001; Yarden and Sliwkowski, 2001]. Modulation of HER2 must be tightly regulated for normal cellular function. In vitro and animal studies clearly demonstrate that deregulated HER2 expression/activity plays a pivotal role in oncogenic transformation, tumorigenesis and metastasis [Hynes and Stern, 1994; Neve et al., 2001; Menard et al., 2003; Citri and Yarden, 2006]. In breast cancer disease, amplification of *HER2* oncogene leading to overexpression of the HER2-coded $p185^{Her\text{-}2/neu}$ oncoprotein occurs in 15–30% of breast carcinomas and associates with unfavourable prognosis, shorter relapse time, and low survival rate [Slamon et al., 1987; Pegram et al., 2000]. Unfortunately, the proteins that regulate the "output" of the HER2 oncogene are not well characterized. Auspiciously, a recent proteomic study has revealed that proteins involved in

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*Correspondence to: Dr. Javier A. Menendez, PhD, Catalan Institute of Oncology, Girona (ICO-Girona), Hospital de Girona "Dr. Josep Trueta", Ctra. França s/n, E-17007 Girona, Catalonia, Spain. E-mail: jmenendez@ico.scs.es

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glycolysis and de novo lipid synthesis are highly expressed in HER2-positive breast carcinomas and in HER2-overexpressing breast cancer cell lines [Zhang et al., 2005].

THE "HER2 → FATTY ACID SYNTHASE (FASN) AXIS": A "LIPOGENIC ADVANTAGE" IN THE PATHOGENESIS OF HUMAN CANCER

Although the cross-talk between HER2 and the sink of energy and/or fuel produced from commonly altered metabolic pathways in breast cancer cells (i.e., aerobic glycolysis—Warburg effect, de novo fatty acid—FA-biosynthesis, etc.), remains to be better defined, a body of evidence strongly supports the notion that the oncogenic effects of HER2 largely depend on the preservation of the "lipogenic phenotype" (i.e., activation of the genetic program involved in de novo FA synthesis including lipogenic enzymes and key metabolic regulators) [Menendez and Lupu, 2004, 2006, 2007; Menendez et al., 2005a,b,c; Vazquez-Martin et al., 2008]. First, HER2 overexpression leads to constitutive up-regulation and maintenance of an exacerbated endogenous FA biogenesis catalyzed by key lipogenic enzymes such as Fatty Acid Synthase (FASN). Second, disturbance of the lipogenic phenotype rapidly *switches-off* the oncogenic activity of the HER2 signaling platform, ultimately resulting in apoptotic cell death of HER2-overexpressing breast carcinoma cells. Third, an exacerbated endogenous FA biosynthesis is sufficient to trigger a breast cancer-like phenotype functionally dependent on HER2 activity. Indeed, endogenous lipogenesis has been suggested to represent a druggable *Achilles heel* in HER2-driven oncogenesis that should open a new window of opportunity for metabolically combating a biologically aggressive subset of HER2-positive breast carcinomas. Accordingly, pharmacological and siRNA-induced FASN inhibition preferentially induces apoptotic cell death of HER2-overexpressing cancer cells [Kumar-Sinha et al., 2003; Menendez et al., 2004a,b]. This requirement of FASN-driven cellular signalling to maintain malignant transformation induced by HER2 overexpression has been confirmed in animal models. In the transgenic *neu*-N-mouse model, in which females develop mammary carcinomas within 300 days, FASN and HER2 have been found to be highly co-expressed in the mammary epithelium [Alli et al., 2005]. Remarkably, pharmacological FASN blockade significantly delays the development of cancer in this model, with some animals never developing overt carcinoma.

FASN AND ADIPOGENESIS

FASN begins to be recognized as a previously unrecognized player on the proliferation and differentiation of adipocyte precursor cells (i.e., adipogenesis). Using the murine 3T3-L1 fibroblastic cell line—a widely used model system to study the mechanisms involved in the adipogenic process—Liu et al. [2004] were pioneers at investigating the influence of pharmacological inhibitors of FASN activity on adipocyte differentiation. Chemically induced FASN blockade prevented the adipogenic conversion of 3T3-L1 fibroblasts in a dose-dependent manner both in the early phases (48 h) and in the latter phases (8 days) of the process. The ability of the chemical FASN blockers to inhibit the adipocyte differentiation process—which was related with a significant down-regulation in the level of Peroxisome Proliferators-Activated Receptor gamma (PPARgamma) mRNA—has been supported by similar experimental findings presented by Schmid et al. [2005]. When FASN signaling was suppressed either pharmacologically or by siRNA during differentiation of 3T3-L1 cells, this was accompanied by reduction of FASN activity, FASN mRNA, mRNA levels of the transcription factors CEBPalpha and PPARgamma, and complete prevention of visible lipid droplet accumulation. These findings suggest that FASN-generated signals may be essential to support preadipocyte differentiation. Indeed, when considering that pharmacological- and siRNA-induced inhibition of FASN efficiently blocks adipocytic differentiation and leads to the reduction of adipocyte number—two parameters determining adipose tissue mass—it is reasonable to suggest that the induction of FASN-catalyzed adipocytic lipogenesis may contribute to obesity. But, is there also a role for HER2 during adipogenesis?

HER2: A NON-ONCOGENIC ROLE IN REGULATING THE ADIPOGENESIS PROCESS?

Recent experimental evidences have revealed that HER2 might indeed function to modulate the adipogenic conversion of preadipocytes. Two opposing hypotheses, however, have been suggested to explain the influence of HER2 on adipocyte differentiation. Pagano and Calvo [2003] firstly shown that the murine 3T3-L1 fibroblastic cell line express both EGFR (HER1) and HER2. They further suggested that modulation of HER2 expression and activity played a fundamental role during proliferation and adipogenic induction of preadipocytes. In their hands, both EGFR and HER2 were found to exhibit a strong up-regulation during the proliferation phase of the cell culture. However, EGFR and HER2 expression was down-modulated once 3T3-L1 cells were induced to differentiate with isobutyl-methylxantine (MIX) and dexamethasone (Dexa), in the presence of 10% FBS. Harrington et al. [2007] pointed out that Pagano and Calvo [2003] incorrectly compared the abundance of EGFR and HER2 receptors in cell lysates containing equivalent protein content. As differentiated adipocytes have two- to threefold more protein per cell than preadipocytes, these authors suggested that EGFR and HER2 should be measured by immunoblotting procedures in growth-arrested preadipocytes and differentiated adipocytes generated from similar cell numbers and not equivalent protein. In their hands, when GADPH was used as a control to show equivalent cell number among samples, EGFR abundance did not significantly change after differentiation; however, Harrington et al. [2007] reported a remarkable increase in HER2 abundance in differentiated adipocytes. Recently, Pagano et al. [2008] reported that down-modulation of HER2 activity is necessary but not sufficient in the differentiation of 3T3-L1 preadipocytes. Using the tyrphostins AG 825 and AG 879 which, by competing with the ATP binding site in the kinase domain efficiently interfere with the activity of HER receptors, they concluded that HER2 blockade inhibited not only 3T3-L1 cells proliferation but further stimulated 3T3-L1 differentiation induced by MIX-Dexa.

Conversely, Harrington et al. [2007] reported that selective inhibition of the HER2 receptor with AG 825 or small interfering RNA (siRNA) pharmacological blockade of HER2 activity efficiently blocked EGF-promoted 3T3-L1 preadipocyte differentiation.

How to reconcile the above mentioned opposing hypotheses for a role of HER2 during adipocyte differentiation? Considering that FASN expression and activity become up-regulated during adipogenic conversion, we recently hypothesized that a "HER2 → FASN lipogenic axis" previously described in cancer cells might also exist during the differentiation of preadipocytes. Moreover, to definitely establish if the discrepancy between the opposing hypotheses for a role of HER2 during adipocyte differentiation related to the experimental approach utilized to compare the abundance of HER2 receptors in undifferentiated and differentiated adipocytes (i.e., cell lysates containing equivalent protein content versus cell lysates generated from similar cell numbers and not equivalent protein) we decided to concurrently monitoring the expression status of the adipogenic marker FASN and of the HER2 TKR in whole 3T3-L1 cultures as well as in individual 3T3-L1 cells using an automated confocal imaging approach. We treated ~80% confluent 3T3-L1 pre-adipocytes (day 0) with the differentiation cocktail (methylisobutyl xanthine + dexamethasone + insulin + rosiglitazone) for 2 days and then changed to serum-containing medium with insulin and the PPARγ agonist rosiglizatone for an additional 2 days. FASN and HER2 expression and sub-cellular compartmentalization in individual 3T3-L1 cells was monitored using an automated imaging platform (BD Pathway™ Bioimager 855). In some cases, high content imaging of whole 3T3-L1 cell cultures growing in individual wells was captured as a 4 × 4 montage. Upon differentiation, 3T3-L1 cells likewise developed microscopically visible lipid droplets starting at day 5. Concomitantly, there was a significant increase in the content of FASN protein in 3T3-L1 cell cultures (Fig. 1a). Indeed, cytoplasmic accumulation of FASN protein was more prominent in individual 3T3-L1 cells exhibiting differentiation features, further supporting the notion that FASN plays a specific role in the process of adipogenic differentiation. These FASN-related adipogenic features peaked at day 9 and slightly decreased after 14-day of culture (Fig. 1b). HER2 expression in 3T3-L1 fibroblasts was assessed using anti-HER2 mouse monoclonal antibody directed against the C-terminal 14 amino acids of the HER2-coded p185$^{Her\text{-}2/neu}$. Almost negative staining of HER2 was observed in undifferentiated control cultures—not subjected to hormonal treatment but processed at the same time as the differentiating cultures—from day 0 to day 14 (Fig. 2a). Remarkably, cell membrane-associated and mainly cytoplasmic expression of HER2 significantly augmented during the differentiation of 3T3-L1 preadipocytes (Fig. 2b). Accumulation of the HER2 TKR in 3T3-L1 cell cultures and in 3T3-L1 individual cells was evident as early as 2 days after addition of differentiation inducers. As observed for the adipogenic marker FASN, HER2 protein accumulation in whole cultures of differentiated 3T3-L1 cells reached a maximum at day 9 (Fig. 2c). Similarly to FASN, cytoplasmic accumulation of HER2 protein was more prominent in individual 3T3-L1 cells exhibiting differentiation features.

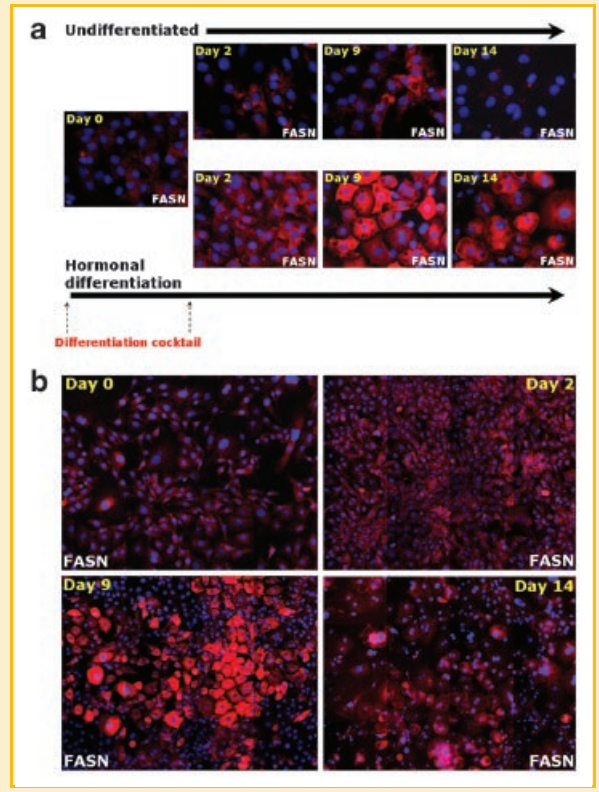

Fig. 1. Monitoring of FASN expression during adipogenic maturation of 3T3-L1 fibroblasts. 3T3-L1 fibroblasts were induced to differentiate by a cocktail of 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM dexamethasone, 1.7 μM insulin, and 1 μM rosiglitazone for 2 days followed by further 2 days treatment with 0.7 μM insulin and 1 μM rosiglitazone and the medium every 2 days. Images show 3T3-L1 cells stained with an anti-FASN mouse monoclonal antibody on days 0, 2, 9, and 14 (a). Cells were prepared using the Triton X-100 permeabilization method and the second step reagent was Alexa Fluor 488® goat anti-mouse IgG as described under Experimental Procedures Section. The images were captured in the two different channels for Alexa Fluor® 488 (pseudo-colored red—FASN) and Hoeschst 33341 (pseudo-colored blue—nuclei) on a BD Pathway™ 855 Bioimager System (Becton Dickinson Biosciences, San Jose, CA) with a 20× objective (NA 075 Olympus) according to the Recommended Assay Procedure and merged using BD Attovision™ software. Images of FASN expression in whole 3T3-L1 cell cultures undergoing the differentiation process were captured as a 4 × 4 montage (b).

HER2 AND ENDOGENOUS LIPID METABOLISM: FROM NORMAL PHYSIOLOGY TO PATHOLOGY

HER2 was first identified as an oncogene due to its ability to induce the malignant transformation of cultured fibroblasts. HER2 oncogene is highly expressed in many cancer types and its overexpression is correlated with a poor prognosis for breast and ovarian cancer patients. As a consequence, a great deal of HER2 signaling research has concentrated on cancer cells ad HER2 has become an important therapeutic target in breast cancer and other human carcinomas for two main reasons: (a) HER2 expression levels closely correlate with the pathogenesis and aggressive behavior of some human carcinomas; and, (b) the level of HER2 in human cancer

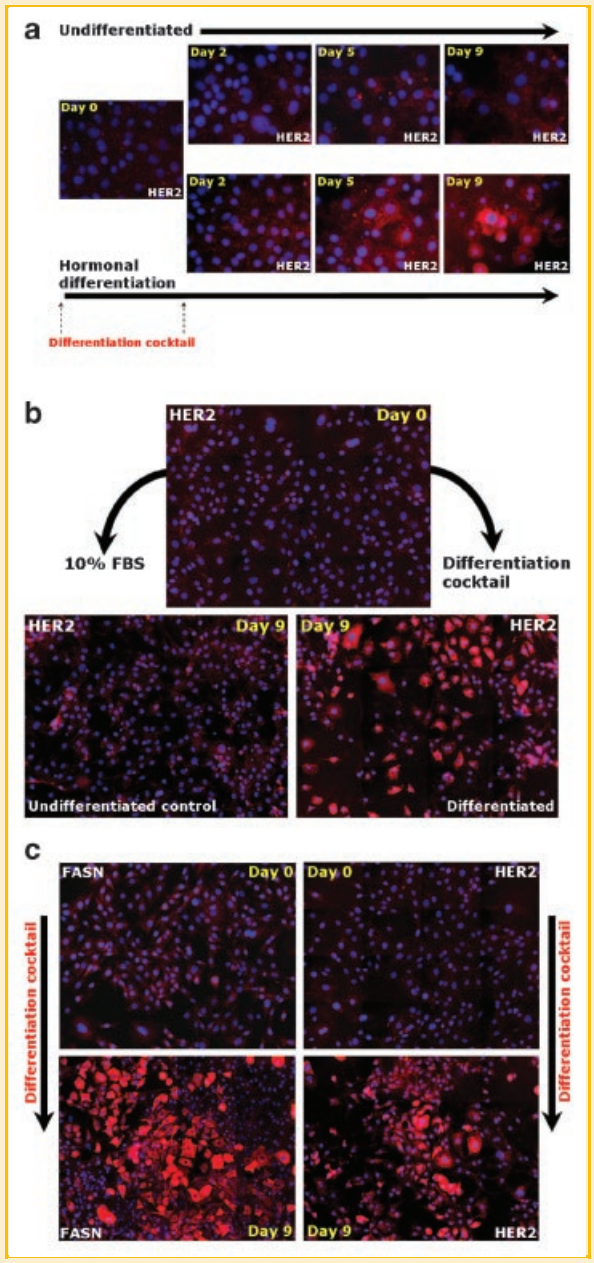

Fig. 2. Monitoring of HER2 expression during adipogenic maturation of 3T3-L1 fibroblasts. 3T3-L1 fibroblasts were induced to differentiate as described in Figure 1. Images show 3T3-L1 cells stained with an anti-HER2 mouse monoclonal antibody on days 0, 2, 9 and 14 (a). Cells were prepared using the Triton X-100 permeabilization method and the second step reagent was Alexa Fluor 488 goat anti-mouse IgG as described under Experimental Procedures Section. The images were captured in the two different channels for Alexa Fluor® 488 (pseudo-colored red—HER2) and Hoeschst 33341 (pseudo-colored blue—nuclei-) on a BD Pathway™ 855 Bioimager System (Becton Dickinson Biosciences) with a 20× objective (NA 075 Olympus) according to the Recommended Assay Procedure and merged using BD Attovision™ software. Images of HER2 expression in whole 3T3-L1 cell cultures undergoing the differentiation process were captured as a 4 × 4 montage (b,c).

cells bearing HER2 gene amplification is much higher than that in normal adult tissues, potentially reducing the toxicity of HER2-targeting drugs. However, little is known about the timing, pattern, and tissue distribution of HER2 and other HER receptors (i.e., HER1, HER3 and HER4), their affinity for HER ligands, and their ability to homo- or heterodimerize to regulate biological responses resulting from distinct HER2-induced signaling pathways in target tissues others than epithelial carcinomas.

HER2 is expressed in multiple neuronal and non-neuronal tissues in embryos and adult animals, including the heart. Genetic data demonstrated that HER2 is required for normal embryonic development of neural crest-derived cranial sensory neurons. HER2-null mutant embryos die before embryonic day 11 of a cardiac trabeculation defect. Upon generation of conditional mutant mice carrying a cardiac-restricted deletion of HER2, we have recently learned that HER2 is a survival factor for human cardiomyocytes, and its inhibition may explain the increased incidence of cardiomyopathy associated with the clinical use of the anti-HER2 monoclonal antibody Tzb, particularly in patients with prior exposure to cardiotoxic chemotherapies, for example, anthracyclines. These studies demonstrated early cardiac dysfunction and severe dilated cardiomyopathy, thus suggesting that HER2 has a role in normal heart physiology as it is essential for cardiac development and function [Crone et al., 2002; Ozcelik et al., 2002; Grazette et al., 2004]. These findings, altogether, clearly expose that HER2 presents a great challenge to devise a selective treatment for a specific cell origin of disease. In this regard, comparative understanding of how HER2 signals in cardiovascular and other cell systems will be important to the development of selective treatment of diseases involving deregulation of HER2 function.

HER2-regulated endogenous metabolism might represent a common molecular feature for both the oncogenic and the natural (physiologic) functions of HER2. (1) Proteomic studies aimed to identify the proteins that are associated with the aggressive phenotype of HER2-positive human cancer revealed that HER2 signaling mainly result, directly or indirectly, in enhanced activation of various metabolic (i.e., glycolysis and lipid synthesis), stress-responsive, antioxidative, and detoxification processes [Zhang et al., 2005]; (2) HER2 overexpression leads to constitutive up-regulation of FASN-catalyzed endogenous FA biogenesis, a "lipogenic benefit" in terms of enhanced cell proliferation, survival, chemoresistance and metastasis in HER2-positive breast carcinoma cells [Menendez and Lupu, 2004, 2006, 2007; Menendez et al., 2005a,b,c; Vazquez-Martin et al., 2008]; (3) HER2 tyrosine kinase activity regulates AMP-activated kinase (AMPK), a well conserved metabolic regulator expressed in all eukaryotes that, upon activation, slow or stop high ATP consuming mechanisms (e.g., FA synthesis) and activate ATP production (e.g., FA oxidation and glycolysis) [Spector et al., 2007; Shell et al., 2008]. Another HER2-regulated "metabolic scenario" seems to be the adipogenic maturation of pre-adipocytes. During puberty and at sexual maturation, when proliferation has decreased in rat mammary glands, Darcy et al. [2000] first revealed that adipocytes surrounding ducts and alveoli expressed abundant HER2 protein. These authors were pioneers at suggesting that non-oncogenic HER2 populations might stimulate lipogenesis in mature adipocytes. On the other hand, Schmid et al. [2005] demonstrated that the up-regulation of FASN during preadipocyte differentiation not only

provides substrates for triacylglycerols synthesis, but that these products are employed to maintain and sustain signaling for the differentiation process. Therefore, FASN is not only an endpoint of preadipocyte differentiation but it is further an active player. Our current findings confirm that FASN is greatly upregulated during pre-adipocyte differentiation. Although Oil-red O staining is frequently used to quantify the extent of differentiation, this has the drawback that it is difficult to characterize the extent of differentiation within the population of treated cells. In our hands, the difference between undifferentiated cells and differentiated cells appeared clearly pronounced when the cells were examined microscopically for FASN immunostaining. Indeed, the majority of FASN-positive cells were, in fact, lipid droplets-positive differentiated cells. Importantly, the occurrence of FASN up-regulation as an early marker of preadipocyte differentiation was concurrently accompanied by up-regulation of HER2. As observed with FASN, HER2 up-regulation was more evident in 3T3-L1 cells developing visible lipid droplets. Although we recently described FASN regulation by HER2 [Menendez and Lupu, 2004, 2006, 2007; Menendez et al., 2005a,b,c] and vice versa [Vazquez-Martin et al., 2008], no cause-effect relationship could still be drawn from the results presented herein. However, preliminary results in our laboratory have revealed that differentiation of 3T3-L1 cells in the presence of either HER2-targeting therapies (e.g., HER1/2 tyrosine kinase inhibitors) or HER2-specific small interfering RNA (siRNA) significantly prevents or significantly reduces the morphological changes observed in untreated (i.e., HER2 active) control differentiated cells. In fact, lipid accumulation and FASN protein content in cells differentiated grown upon HER2 blockade resemble those of undifferentiated cells [Vazquez-Martin et al., unpublished observations]. That is, when added at an early phase of the differentiation process, pharmacological- and siRNA-induced inhibition of HER2 appears to prevent FASN up-regulation in differentiated adipocytes and, consequently, to repress the differentiation of 3T3-L1 cell cultures. The fact that the adipogenic inducer Dexa significantly interferes with the growth inhibitory actions of the anti-HER2 monoclonal antibody in HER2-overexpressing breast cancer cells [Sumikawa et al., 2008] does not support a HER2 downmodulating effect of this glucocorticoid during hormonal induction of adipogenesis [Pagano and Calvo, 2003].

Our current findings, altogether, confirm the notion that the HER2 TKR might play a previously unrecognized non-oncogenic role in regulating the adipogenesis process through its ability to control the expression of the adipogenic enzyme FASN. These results further suggest that inhibition of preadipocyte differentiation, and the possible reduction of adipose tissue, might represent an exploitable molecular avenue of using inhibitors of the HER2 → FASN axis as a therapy for the treatment of obesity.

EXPERIMENTAL PROCEDURES

CELL CULTURE

3T3-L1 fibroblasts were obtained from the American Type Culture Collection (ATCC) and were used at passage 4–12. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% HEPES (25 mM), 1% Penicillin (100 U/ml)/Streptomycin (100 μg/ml) and 1% L-glutamine (2 mM) at 37°C in the presence of 5% CO_2. Medium was changes every 3 days. For differentiation, 3T3-L1 fibroblasts were seeded at approximately 5,000 cells/well in 96-well clear bottom imaging tissue culture plates (BD Biosciences, San Jose, CA; Cat. No. 353219) optimized for automated imaging applications. At 80% confluence, the medium was changed to DMEM containing 10% FBS supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM dexamethasone, 1.7 μM insulin, and 1 μM rosiglitazone (Differentiation Medium 1, DM1; day 0). Two days later, medium was replaced by DMEM containing 10% FBS, 1.7 μM insulin and 1 μM rosiglitazone (Differentiation Medium 2, DM2; day 2). Medium was then replaced by DMEM containing 10% FBS every 2 days and grown until indicated.

FIXATION

Culture media were removed from well and freshly prepared 3.7% formaldehyde (Sigma, St. Louis, MO, F-1268) diluted in 1× phosphate buffered saline (PBS) and pre-warmed to 37°C was added (100 μl/well). Following incubation at room temperature (RT) for 10 min, the fixation solution was removed and the residual liquid was removed by inverting the plate briefly onto absorbent paper. Plates were washed twice by adding 100 μl/well of 1× PBS.

TRITON X100 PERMEABILIZATION AND BLOCKING

1× PBS was removed and 100 μl/well of a 0.1% Triton® X-100 (Sigma, T-9284) solution diluted in 1× PBS were added. Following incubation at RT for 5 min, the permeabilization solution was removed and the residual liquid was drained. Plates were washed twice by adding 100 μl/well of 1× PBS. 1× PBS was removed and 100 μl/well of 1× PBS supplemented with 5% FBS were added. Following incubation at RT for 30 min, we proceeded to the Primary Antibody Staining step.

PRIMARY ANTIBODY STAINING

Once the blocking solution was removed, 50 μl/well of primary antibodies (2.5 μg/ml of the anti-FASN mouse monoclonal antibody—BD Biosciences, Clone 23; Cat. No. 610962 and 2.5 μg/ml of the anti-c-*erb*B-2/c-*neu* Ab-3 mouse monoclonal antibody—Calbiochem, a brand of EMD Chemicals, Inc., Gibbstown, NJ, Clone 3B5; Cat. No. OP-15) diluted to the appropriate concentration in blocking solution were added. Following incubation at RT for 1 h, primary antibody solution was removed and plates were washed three times with 1× PBS before proceeding with the Secondary Antibody Staining.

SECONDARY ANTIBODY STAINING

1× PBS was removed and 50 μl/well of diluted secondary antibody was added. Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, OR; Cat. No. A11029) antibody was diluted in blocking solution to 0.05 mg/ml. Following incubation at RT for 1 h in the dark, secondary antibody solution was removed and plates were washed three times with 1× PBS. 100 μl/well of 1× PBS containing 2 μg/ml Hoechst 33342 (Invitrogen, Cat. No. H3570) were added, the plate was covered and let stand protected from light for 15 min. Plates were immediately imaged or stored at 4°C in the dark prior to imaging.

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