

ORIGINAL ARTICLE

Breast cancer dissemination promoted by a neuregulin-collagenase 3 signalling node

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Advances in the treatment of breast cancer have resulted in increased survival. However, in the metastatic setting, the disease remains incurable. Therefore, understanding of the mechanisms that promote dissemination of breast cancer cells may favor the development of novel therapeutic strategies to fight those tumors. Here, we show that the ErbB ligands, Neuregulins (NRGs), promote metastatic dissemination of breast cancer cells by switching on a kinase-metalloproteinase network. Clinicopathological analyses demonstrated that NRG expression in breast tumors associated to lymph node invasion and poor patient outcome. Preclinical *in vivo* analyses showed that NRG expression favored *in situ* tumor growth, local spreading and metastatic dissemination. Genomic, biochemical and functional studies identified matrix metalloproteinases, particularly stromelysin 2 and collagenase 3, as key mediators of the NRG-induced dissemination properties of breast cancer cells. Mechanistic analyses demonstrated that NRG augmented metalloproteinase expression through a route controlled by ERK1/2 kinases. ERK1/2 increased collagenase 3 expression by controlling the activity of an SBF1-related transcription factor. In conclusion, we describe a pathway linked to breast cancer dissemination. The clinical availability of agents that target some of the components of this signalling pathway suggests that patients with tumors fed by NRGs or other factors able to activate the ERK-Collagenase 3 route may benefit from agents that act on that signalling axis.

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INTRODUCTION

Metastatic dissemination of cancer cells is a hallmark of malignization.¹ Despite relevant advances in this field, therapeutic agents aimed at inhibiting the metastatic capabilities of cancer cells have not reached the clinical setting, especially because of the still limited knowledge about metastatic mechanisms and actionable molecular targets. Therefore, the discovery of biological programs linked to metastatic spreading that will inform about the risk of tumor relapse and worse outcome is a key objective in cancer research.²

A prototypical example of a tumor whose life-threatening properties depends on metastatic dissemination is breast cancer. Among the several molecular types of breast cancer, one of them, characterized by overexpression of the transmembrane tyrosine kinase HER2, has been linked to worse patient outcome owing to frequent metastatic dissemination.^{3–5} Because of this, therapies that target HER2 have been developed with the purpose of reducing the prooncogenic activity of HER2.^{6,7} Such therapies include monoclonal antibodies that interact with the extracellular domain of HER2, such as trastuzumab, its derivative trastuzumab-emtansine, or pertuzumab.⁸ In addition, the small HER2 tyrosine kinase inhibitor lapatinib has also reached the breast cancer clinic.⁷

Although most studies have focused on HER2 overexpression as a driver of metastatic breast cancer, alternative modes of HER2 activation which could also promote metastatic dissemination have been less well studied. However, various evidences indicate that ligand-mediated activation of HER2 may also contribute to the pathophysiology of breast tumors, especially in situations in which HER2 is not overexpressed. Thus, preclinical studies

indicated that increased expression of the HER2-activating ligands, neuregulins (NRGs), in the mammary gland of mice provoked the appearance of adenocarcinomas.⁹ Moreover, augmented expression of NRGs has been reported to favor the metastatic spread of breast cancer cells in preclinical models.^{10,11} *In vitro*, NRGs act as potent stimulators of proliferation,^{12,13} and this effect can be counteracted by trastuzumab,¹⁴ indicating that participation of HER2 is important in the mitogenic response to NRGs. Importantly, NRGs have also been linked to resistance to anti-HER2 therapies,¹⁵ potentially through mechanisms that may involve protection of tumor-initiating cells.¹⁶

In addition to these preclinical studies, increasing evidence indicates that NRGs may also contribute to the pathogenesis of human breast cancer. NRGs are expressed in a number of breast cancer samples.^{17,18} Moreover, several studies have indicated that targeting of the NRG-HER system may improve clinical outcome in breast cancer, especially in patients with HER2-low tumors.¹⁹ Thus, a clinicopathological study revealed that breast cancer patients whose tumors expressed NRG benefited from trastuzumab therapy, even in the absence of HER2 overexpression.¹⁷ Furthermore, the B-31²⁰ and the N9831²¹ clinical trials observed that addition of trastuzumab to standard-of-care treatment regimens benefited patients with HER2-low tumors. As a consequence of these studies, the ongoing NSABP B-47 trial is being carried out to test the efficacy of trastuzumab addition to standard chemotherapy in patients with HER2-low tumors.

To better understand how NRGs contribute to the pathophysiology of breast cancer, we evaluated the clinical relevance of NRG expression in a subset of breast cancer patients. These

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studies indicated that NRG expression was associated with a worse outcome owing to increased metastatic dissemination of tumors expressing NRG. We then undertook a genomic approach to identify signalling mediators that drive such dissemination. We identified collagenase 3 as an important mediator of NRG signalling in breast cancer, and have found that the expression of collagenase 3 facilitates the spreading of tumoral cells upon activation of NRG receptors. Mechanistic studies defined the pathway that controlled collagenase 3 expression by NRG. We present data that support that targeting of collagenase 3 may be useful to prevent metastatic dissemination of breast cancer cells. In conclusion, we describe a druggable pathway linked to breast cancer dissemination that can be recognized in patients by evaluating NRG expression in their primary tumors.

RESULTS

NRG expression linked to poor patient outcome

The association between NRG expression and breast cancer outcome was evaluated in a cohort of 76 patients whose clinicopathological characteristics are described in Supplementary Table 1. NRG expression in patient samples was analyzed by immunohistochemistry using an antibody that recognizes the intracellular region of transmembrane NRGs (Supplementary Figure S1A), and offers a membrane staining pattern (Figure 1a).²² Western blotting verified that the antibody specifically recognized several NRG forms in patient samples (Supplementary Figure S1B). Twenty-six (34.2%) of the 76 tumors analyzed were positive for

NRG staining. Patients bearing tumors positive for NRG had a worse disease-free survival (DFS) than those being negative for NRG (Figure 1b). Moreover, higher amounts of NRG were associated with increased risk of relapse (Figure 1c). As spreading to lymph nodes represents an initial step in the metastatic process, we evaluated the expression of NRG in tumoral axillary lymph nodes as well as at the primary tumor site. A statistically significant correlation was observed between NRG expression in primary tumors and axillary lymph nodes infiltrated by the tumoral cells (Figure 1d). Together, these clinicopathological data indicated that NRG expression was associated with an increased risk of tumor relapses as shown by a worse DFS, which can potentially be caused by the metastatic dissemination trend of the NRG-expressing primary tumors.

NRG expression favors *in situ* tumor growth, local spreading and metastatic dissemination

To explore how the expression of NRGs may translate into worse clinical outcome, we used preclinical models based on the MCF7 cells, which respond mitogenically to NRG as they express NRG receptors.¹³ Parental as well as MCF7 cells engineered to express the transmembrane NRG isoform proNRGα2c (MCF7-NRGα2c) were used.¹⁴ In addition, MCF7 and MCF7-NRGα2c cells were transfected with a luciferase reporter which allowed the visualization of tumor masses *in vivo*. MCF7-NRGα2c cells proliferated better than wild-type MCF7 cells *in vitro* (Figure 2a). Biochemically, this was accompanied by the constitutive activation of signal

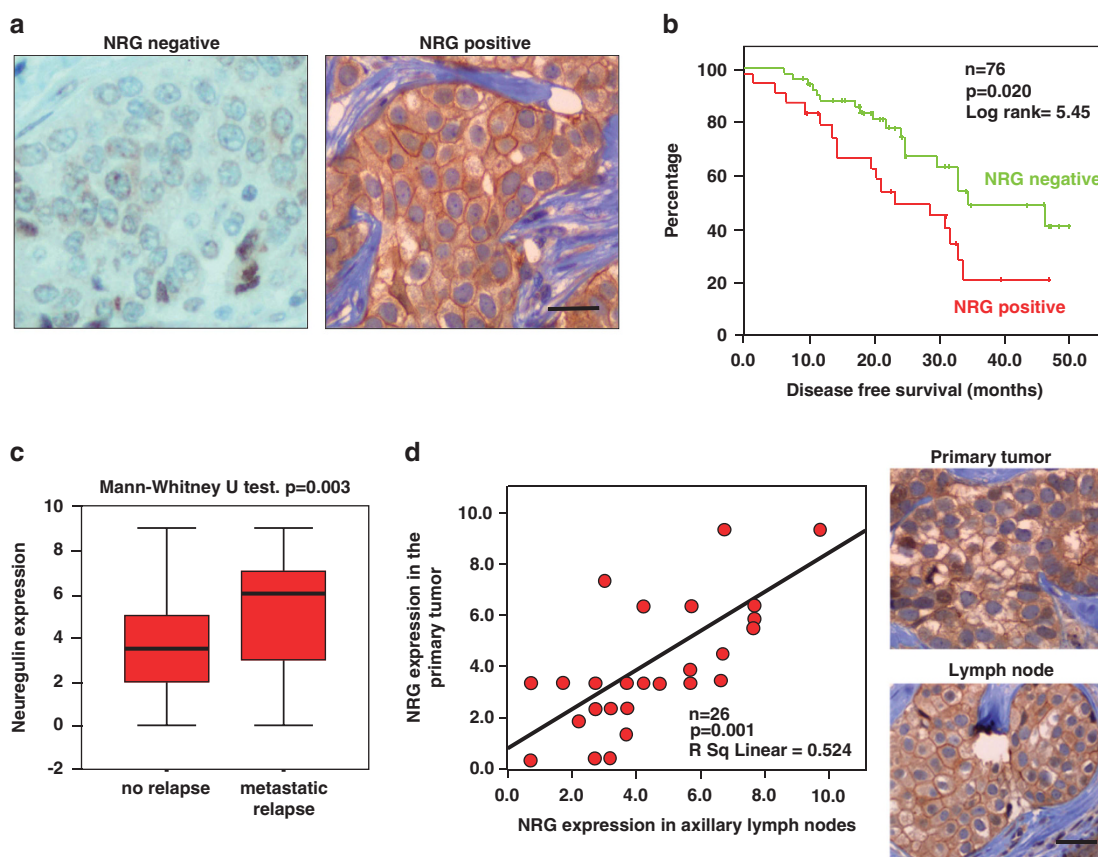


Figure 1. NRG expression is linked to poor patient outcome. (a) Immunohistochemical staining of two breast tumors (one considered negative and one considered positive) using the anti-NRG antibody. Scale bar = 40 μ m. (b) Kaplan-Meier analysis of the relationship between expression of NRG and DFS in 76 patients. (c) Mann-Whitney analysis showing the link between NRG expression in the primary tumor and metastatic relapse. (d) Regression analysis illustrating the relationship among the levels of NRG in the primary tumor and lymph nodes. Immunohistochemical examples of NRG staining in the primary tumor and metastatic lymph node are shown at the right. Scale bar = 40 μ m.

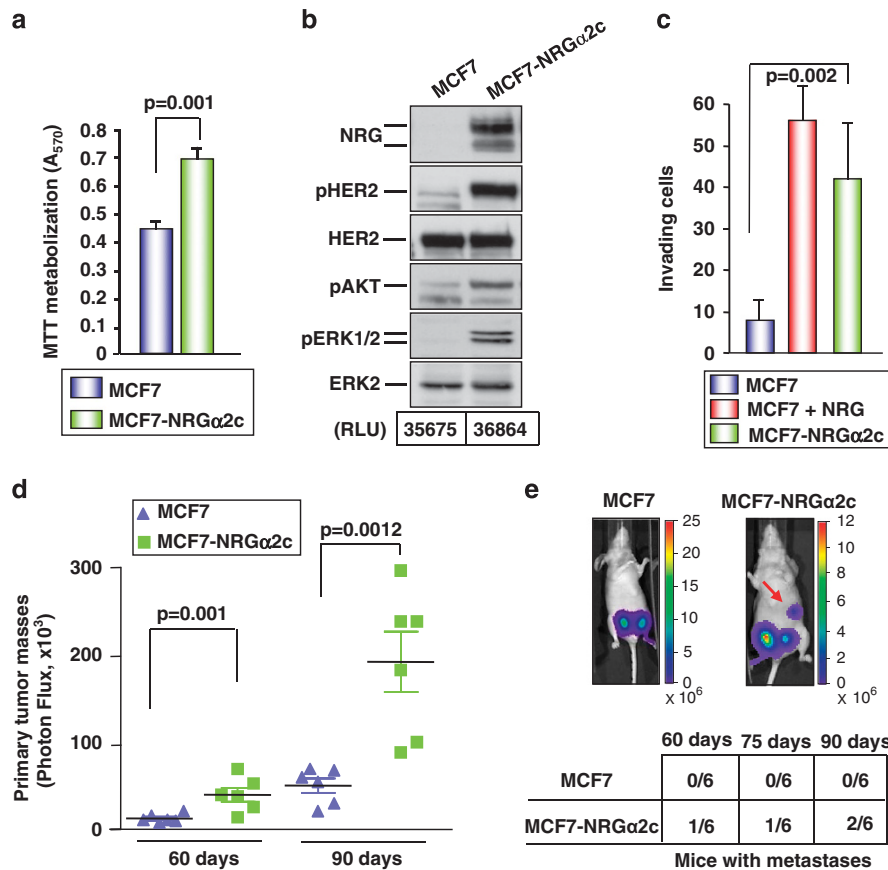


Figure 2. NRG expression favors tumor growth and spreading. **(a)** *In vitro* analysis of the effect of NRGα2c expression on cell proliferation. A total of 25 000 MCF7 or MCF7-NRGα2c cells were plated in each well and cell proliferation measured by MTT metabolism. Results are plotted as the raw absorbance values, and represent the mean \pm s.d. of quadruplicates. **(b)** Signalling status of the NRG-ErbB pathway in MCF7 or MCF7-NRGα2c cells. Analyses of the different signaling intermediates was performed by western blotting of cell extracts prepared from luciferized MCF7 or MCF7-NRGα2c cells. The values at the bottom represent luminescent readings (RLU) from both cell lines. **(c)** Superior invasiveness properties of MCF7 cells expressing NRGα2c as compared with wild-type MCF7 cells. A total of 20 000 cells were plated on top of a matrigel cushion lied on transwells. Culture media was added at the bottom of the well. In the case of MCF7 cells, the culture media of some samples included NRG (10 nM, column 2). Forty-eight hours later, invading cells were identified by crystal violet staining and counted. Results are plotted as the mean \pm s.d. of quadruplicates. **(d)** Tumor mass analyses of mice injected with MCF7 or MCF7-NRGα2c cells. Photon fluxes at 60 and 90 days were taken using an IVIS50 apparatus, and data quantitated using the Igor Pro 4.0 software (Xenogen). **(e)** Luminescence analyses of mice injected with MCF7-Luc or MCF7-NRGα2c-Luc cells. Pictures were taken 90 days after injection. The rainbow bar at the right of each image represents the intensity scale of the luminescence signal. The arrow points to a disseminated lesion. The table at the bottom indicates the number of mice with metastasis of a total of six mice used in each of the analyzed groups.

transduction routes linked to cell proliferation, as demonstrated by increased phosphorylation of HER2, AKT at Ser⁴⁷³ and ERK1/2 at their TEY activation microdomain (Figure 2b).

MCF7 cells were poorly invasive (Figure 2c). However, when MCF7 cells were treated with exogenous soluble NRG or were forced to express transmembrane proNRGα2c, they invaded at much higher rates than parental untreated MCF7 cells (Figure 2c). We explored the behavior of the NRG-expressing MCF7 cells *in vivo* by orthotopic implantation of parental MCF7 or MCF7-NRGα2c cells in the caudal mammary fat pad of mice, at two sites per mouse. Tumors derived from MCF7-NRGα2c cells were larger than those generated by parental MCF7 cells (Figure 2d). Also, local spreading of the tumors was more evident in mice injected with MCF7-NRGα2c cells (Figure 2e). Moreover, tumors from mice injected with MCF7-NRGα2c cells had a tendency to expand to new sites beyond the site of implantation of the cells (Figure 2e).

Gene expression profiling identifies matrix metalloproteinases as effectors of NRG signaling

To gain insights into the mechanism responsible for the protumorigenic actions of NRG, we performed gene expression

analyses of MCF7 cells treated with soluble NRG for 3, 6, 12 and 24 h. Gene expression data from three independent experiments were obtained, and genes whose levels changed twofold or more in all three experiments were considered for further analyses. Functional categorization indicated that NRG stimulation caused changes in genes associated with nuclear receptor pathways (retinoic acid and glucocorticoid receptors), and in the transforming growth factor- β and integrin and metalloprotease categories (Supplementary Figure S2A). Because of the involvement of the latter three categories in the regulation of dissemination of breast cancer cells,²³ we decided to further validate at the mRNA and protein levels the genes deregulated by NRG treatment and included in those three categories. These experiments confirmed upregulation or downregulation of several of the genes identified in the gene expression analyses (Figures 3a and b, and Supplementary Figure S2B). Especially important was the upregulation of collagenase 3,²⁴ a protease formerly linked to bone metastases and poor prognosis in breast cancer.^{25,26} Collagenase 3 levels were rapidly increased by NRG treatment of MCF7 cells, being detected in cell lysates at 2 h of treatment with the growth factor (Figure 3c). The collagenase was released into the culture media where it accumulated (Figure 3c), and its activity

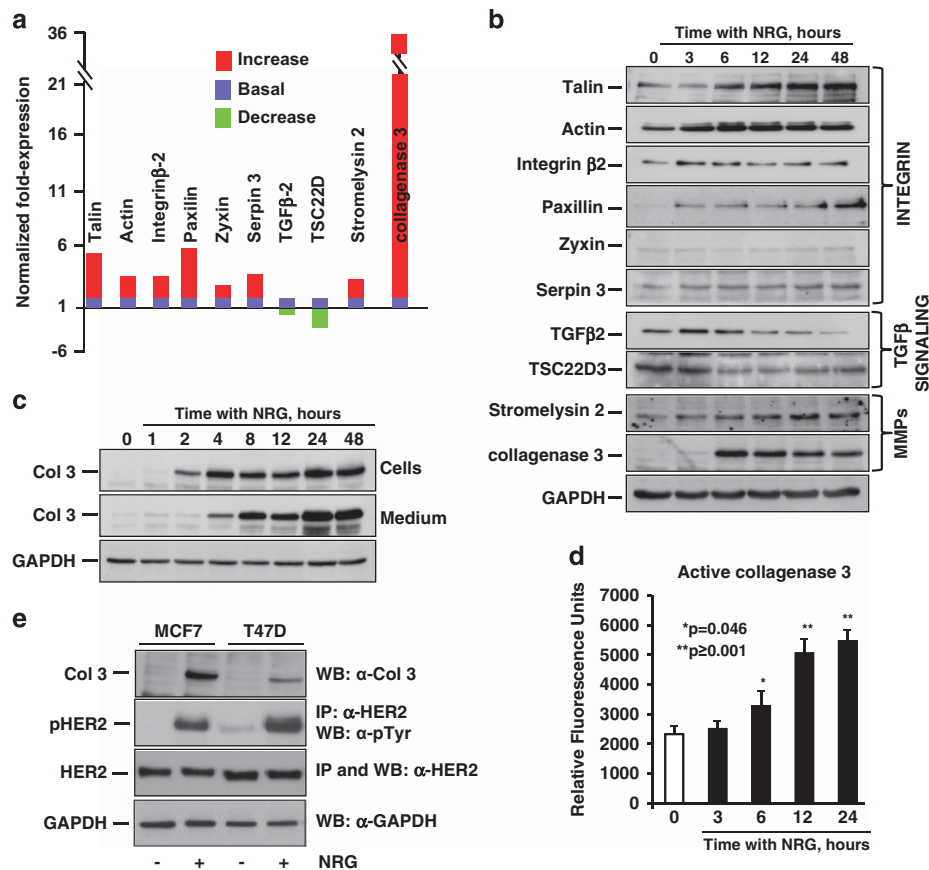


Figure 3. Major gene categories deregulated by NRG in MCF7 cells. **(a)** qPCR analyses of genes included in major pathways deregulated by NRG. MCF7 cells were treated with NRG (10 nM) for different times (3, 6, 12, 24 h, shown only the 24 h time-point) and expression analyses performed. Changes in expression with respect to the resting level (basal = 1) are plotted. **(b)** Western blot analyses of the proteins affected by NRG treatment of MCF7 cells. MCF7 cells were treated with NRG (10 nM) for the indicated times. Fifty micrograms of protein extracts were loaded in each lane, and blots probed with antibodies against the indicated antigens. **(c)** Time-course analyses of the effect of NRG on the expression of collagenase 3. Exponentially growing MCF7 cells were washed twice with serum-free culture media and treated with NRG at different times. Culture media were collected, and cells were lysed. Collagenase 3 levels in cells were analyzed by western blotting using 50 μ g of the cell lysates and the collagenase 3 antibody. Collagenase 3 in media samples was analyzed after immunoprecipitation and western blotting with the same antibody. **(d)** Release of active collagenase 3 by MCF7 cells upon treatment with NRG. Cells were treated with 10 nM NRG for the indicated times, and collagenase 3 activity in the culture media analyzed using a fluorescent enzymatic assay. Results are shown as the mean \pm s.d. of triplicates. *P* indicates significance values with respect to resting measurements of activity. **(e)** MCF7 or T47D cells were serum-starved for 12 h and then treated for 6 h with 10 nM NRG. Analyses of collagenase 3 and GAPDH expression were directly performed on cell extracts, while that HER2 and pHER2 were carried out after immunoprecipitation using anti-HER2 antibodies.

increased over time (Figure 3d). As expected, the accumulation of collagenase 3 in the culture media was delayed with respect to its accumulation in the cells.

Whether upregulation of collagenase 3 production upon activation of HER receptors was a general phenomenon was next explored. To this end, we used the T47D cell line, which shares several characteristics with MCF7, such as low HER2 expression and ability to mitogenically respond to NRG. Treatment with NRG provoked the accumulation of collagenase 3 in the T47D cells (Figure 3e).

Collagenase 3 facilitates dissemination of breast cancer cells

To evaluate the participation of collagenase 3 in proliferation or metastatic dissemination of breast cancer cells upon stimulation by NRG, we knocked down the expression of collagenase 3 using short hairpin RNA. From the five short hairpin RNA vectors used, two of them caused substantial knockdown of collagenase 3, and gave analogous results although data with only one of them are presented. NRG-induced invasion (Figure 4a), cell adhesion

(Figure 4b) and wound repair (Figure 4c) were compromised in collagenase 3-knocked down cells.

Luciferized MCF7-NRGa2c cells were used to explore the *in vivo* effect of collagenase 3 knockdown. *In vitro* biochemical experiments evidenced that knocking down collagenase 3 did not affect NRG-HER receptor signaling in cells with similar levels of luciferase activity (Supplementary Figure S3 and data not shown). Injection of MCF7-NRGa2c or MCF7-NRGa2c-shcollagenase 3 cells into the breast tissue of mice created tumors at the site of injection (Figure 4d). However, whereas MCF7-NRGa2c presented ectopic luminescence signals, indicative of tumor dissemination, the ones injected with MCF7-NRGa2c-shcollagenase 3 cells did not show expansion of the tumoral mass beyond the sites of injection (Figure 4d). In addition, the tumors from MCF7-NRGa2c showed signs of higher local spreading as compared with tumors created by the MCF7-NRGa2c-shcollagenase 3 cells, as indicated by measurements of the width of the luminescent signal (Figure 4e). Western blotting of tumoral samples obtained from both subsets of mice confirmed the downregulation of

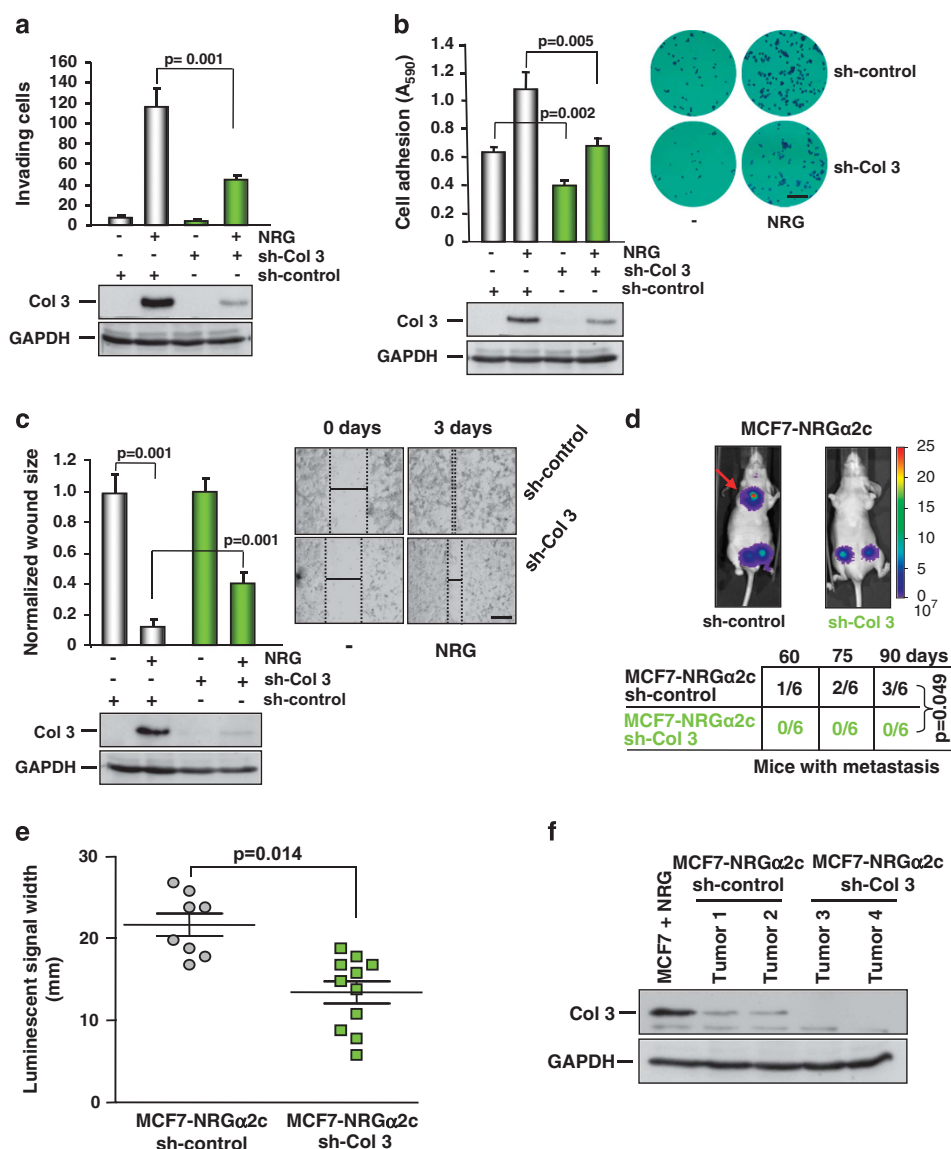


Figure 4. Collagenase 3 mediates NRG-induced dissemination of breast cancer cells. (**a–c**) Effect of collagenase 3 knockdown on NRG-induced invasion (**a**), adhesion (**b**) and wound healing (**c**). MCF7 cells transfected with a control short hairpin or a short hairpin against collagenase 3 were treated with or without 10 nM NRG. The number of invading cells (**a**) was measured 3 days later. In (**b**), cells were plated on fibronectin and cell adhesion was analyzed 2 h later. Representative images of adherent cells (**b**) or wound-healing experiments (**c**) are shown at the right. During wound-healing experiments cultures were treated with mitomycin C (1 μ M). Scale bar in (**b**) and (**c**) = 100 μ m. Results are shown as the mean \pm s.d. of quadruplicates. Western analyses of collagenase 3 and GAPDH levels in MCF7 cells transfected with a control short hairpin or a short hairpin against collagenase 3 are shown. (**d–f**) Xenograft mice model and analysis of tumor width in mice injected with 5×10^6 MCF-7 α 2c-sh-control or MCF-7 α 2c sh-collagenase 3 cells. Images of tumoral masses in both types of xenografts are shown in (**d**), as well as indication of the number of mice with metastasis in each experimental condition. Tumor width was measured using the Living Image software and compared (day 90) using two-sided Student's *t*-test. Mean values \pm s.d. are shown (**e**). (**f**) Analysis of collagenase 3 in tumor xenograft samples. GAPDH was used as a loading control.

collagenase 3 in the samples derived from mice injected with MCF7-NRGα2c-shcollagenase 3 cells (Figure 4f).

The ERK1/2 route mediates the effects of NRG on collagenase 3 expression

To gain insights into the mechanism involved in collagenase 3 upregulation upon activation of NRG receptors, antibody arrays which detect 43 different proteins used as readouts of the activation status of relevant signaling routes were used. In MCF7 cells, these experiments showed upregulation of the activation status of several of these proteins upon treatment with NRG

(Figures 5a and b). Particularly strong was the induction of activating phosphorylations of ERK1/2 and AKT. Because of these findings, and of the important role of the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR pathways in signal transduction, we explored the contribution of both pathways in mediating the upregulation of collagenase 3 upon stimulation of NRG receptors. The MEK inhibitor U0126 inhibited collagenase 3 upregulation by NRG in MCF7 cells (Figure 5C). This inhibitor also prevented the phosphorylation of ERK1/2 upon stimulation with NRG. However, the PI3K inhibitor LY294002 did not affect NRG-induced upregulation of collagenase 3, while it inhibited the phosphorylation of AKT at Ser⁴⁷³. The mTOR inhibitors rapamycin and Ku0063794 were

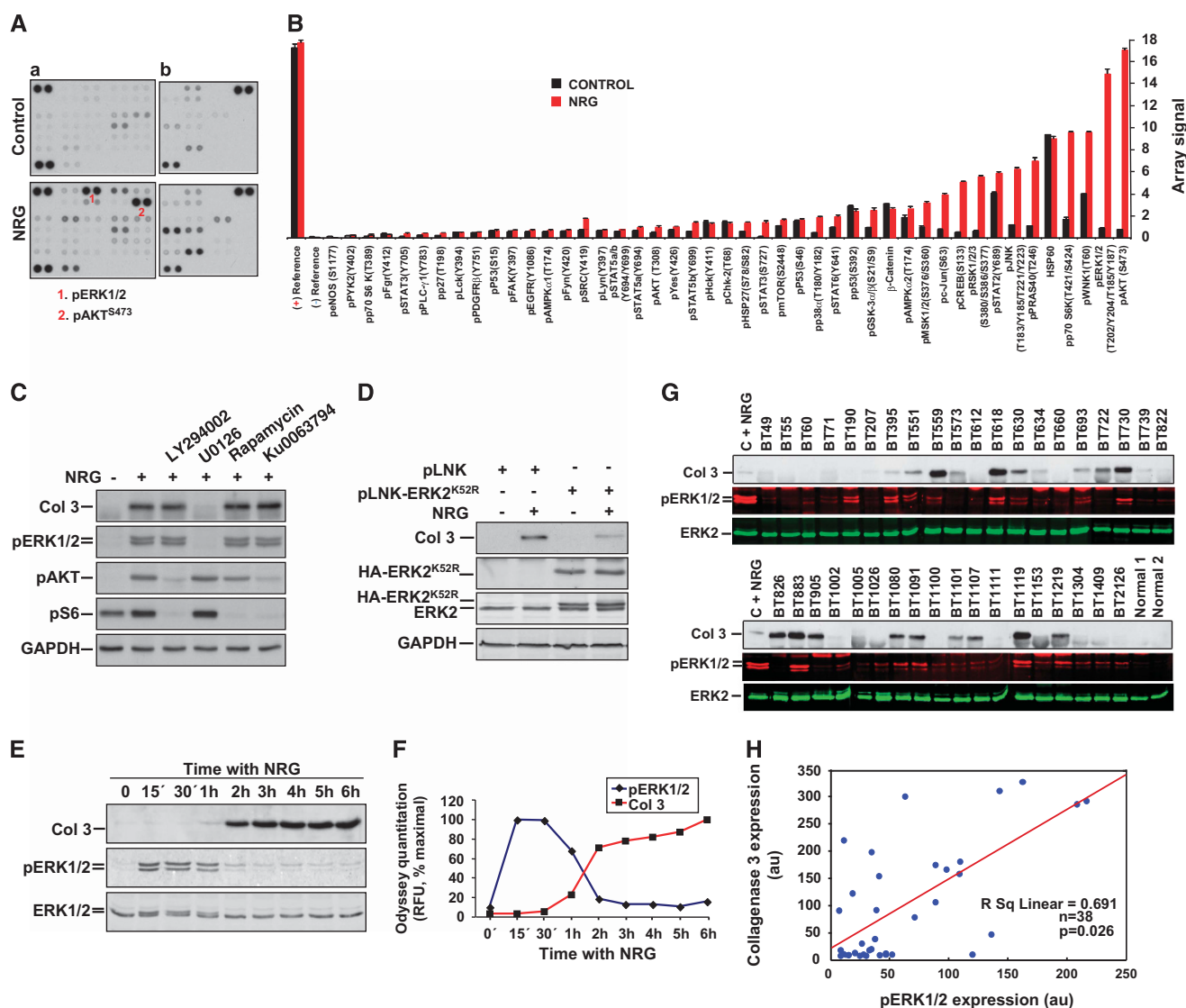


Figure 5. NRG controls collagenase 3 levels through the ERK1/2 route. **(A)** Expression of different antigens used as readouts of activation of different signalling routes. Lysates from MCF7 cells treated or not with NRG were incubated with the antibody arrays to detect active (phosphorylated) forms of different antigens which participate in relevant signalling pathways. **(B)** Quantitation of the array data shown in **(A)**. **(C)** Effect of blockade of the PI3K, mTOR and MEK1/2 on NRG-induced collagenase 3 upregulation. MCF7 cells were preincubated for 1 h with LY294002 (10 μ M), U0126 (10 μ M), Rapamycin (100 nM) or Ku0063794 (1 μ M), and then treated with NRG (10 nM). The levels of collagenase 3, pERK1/2, pAKT, pS6 and GAPDH were analyzed by western blotting. **(D)** Effect of a dominant-negative form of ERK1/2 on NRG-induced upregulation of collagenase 3. MCF7 cells transfected with the vector pLNK-ERK2^{K52R} coding for a dominant negative form of ERK1/2 (HA-tagged ERK2^{K52R}) were incubated with NRG and collagenase 3 expression, ERK1/2, HA-ERK2^{K52R} and GAPDH analyzed in 50 μ g of cell extracts. **(E)** Time course analysis of the effect of NRG on collagenase 3 production, and pERK1/2 and ERK1/2 levels. Fifty micrograms of protein from each sample were used in western blotting. **(F)** Quantitative analyses of the collagenase 3 and pERK1/2 data shown in **(E)**. **(G)** Western blotting analyses of collagenase 3, pERK1/2 and ERK1/2 expression in patient samples. Collagenase 3 was immunoprecipitated from 2 mg of protein. Assessment of pERK1/2 and ERK1/2 was carried out directly on 50 μ g of protein from each sample. **(H)** Quantitative regression plotting of the data obtained in panel **(G)**.

also unable to prevent NRG-induced upregulation of collagenase 3, or the phosphorylation of ERK1/2. However, they efficiently prevented S6 phosphorylation, which is used as readout of mTORC1 complex activity.²⁷ That ERK1/2 was involved in NRG-induced upregulation of collagenase 3 was also evidenced by using ERK2^{K52R}, a dominant-negative mutant form of ERK2. Transfection of MCF7 cells with an HA-tagged version of ERK2^{K52R} decreased the amount of collagenase 3 released to the culture media of NRG-treated MCF7 cells (Figure 5D). Accumulation of collagenase 3 in the culture media was delayed with respect to the action of NRG on the dual phosphorylation of ERK1/2

(Figures 5e and f), indicating that ERK1/2 activation must precede rises in collagenase 3 released to the extracellular milieu.

We explored the action of other agents on collagenase 3 expression. IGF-1, which activated the AKT route but had a poor effect in the ERK1/2 pathway failed to stimulate collagenase 3 upregulation (Supplementary Figure S4A). On the other hand, treatment with the tumor promoter phorbol-12-myristate, 13-acetate, known to activate several PKC isoforms and indirectly activate ERK1/2,²⁸ caused the accumulation of collagenase 3 in the culture media of MCF7 cells with a similar time course to that obtained by NRG stimulation (Supplementary Figure S4B).

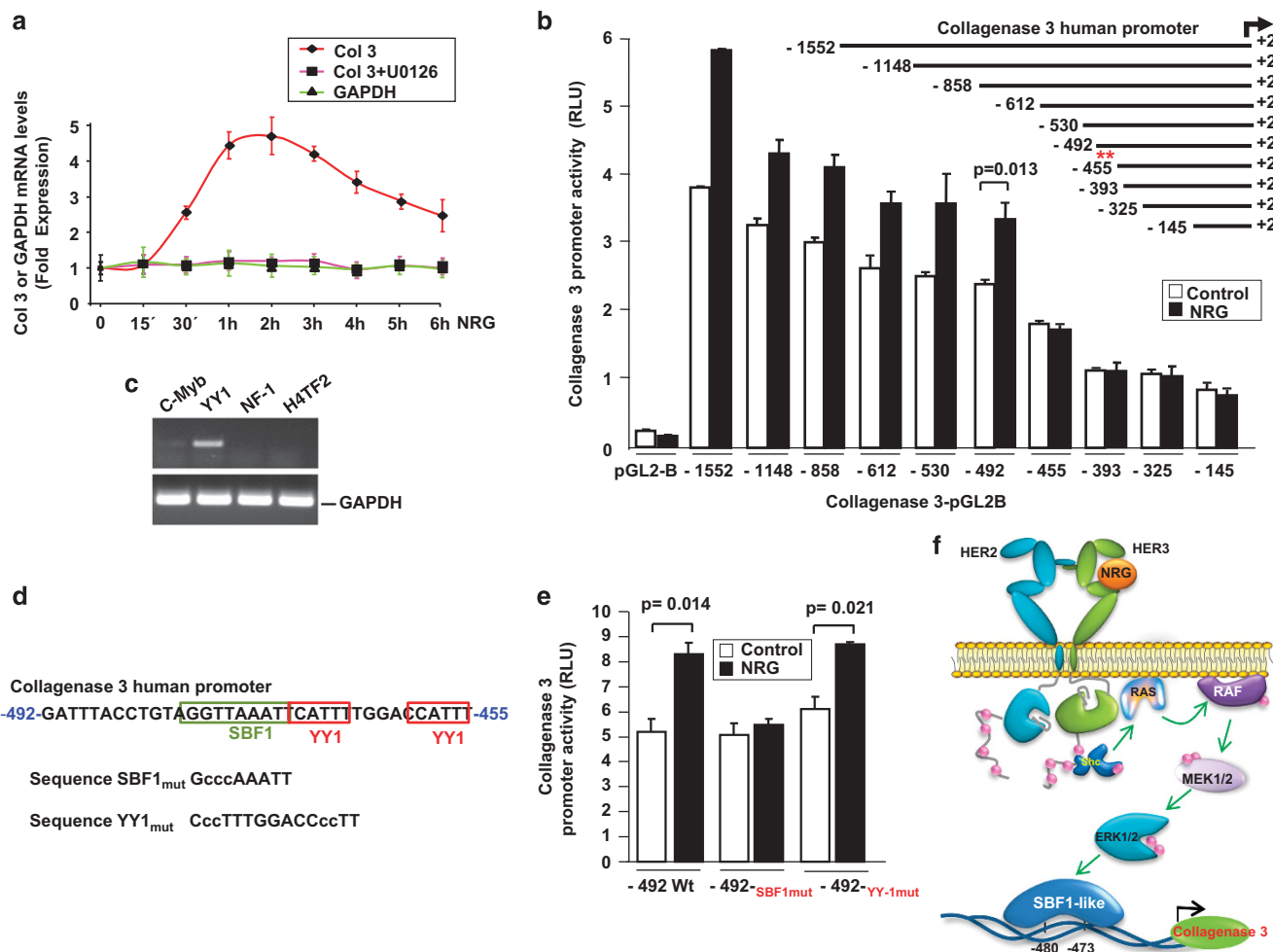


Figure 6. Transcription-dependent upregulation of collagenase-3 levels by NRG. **(a)** qPCR analysis of collagenase 3 mRNA levels in MCF7 cells treated for different times with NRG. A set of samples were preincubated with 10 μ M of the MEK1/2 inhibitor U0126. **(b)** Transcriptional analysis of the collagenase 3 promoter. MCF7 cells were transfected with 1 μ g of each of the reporter plasmids indicated together with 100 ng of the pCDN3-renilla plasmid (used to normalize for transfection efficiency). Luminescent data are presented as mean \pm s.d. **(c)** RT-PCR analyses of the expression of distinct transcription factors in MCF7 cells. **(d)** Description of the promoter region mainly responsible for the regulation of collagenase 3 by NRG. Sequences putatively recognized by the transcription factors YY1 and SBF-1 are shown, as well as the mutant sequences used to analyze the relevance of these regions in the regulation of collagenase 3 expression. **(e)** Identification of the region responsible for the upregulation of collagenase 3 expression upon activation of NRG receptors. MCF7 cells were cotransfected with 1 μ g of the indicated plasmids corresponding to wild-type -492+2 collagenase 3 pGL2-B or SBF-1 and YY1 mutants, together with 100 ng of pCDNA3-renilla. Data are plotted as in **(b)**. **(f)** A schematic representation of the mechanism of activation of collagenase 3 by NRG.

Phorbol-12-myristate,13-acetate did not activate AKT phosphorylation in MCF7 cells.

We used a subset of patient-derived breast tumors to evaluate a potential linkage between activation of ERK1/2 and collagenase 3 expression. Breast tissue obtained from reduction mammoplasties showed low levels of collagenase 3 expression in the normal mammary gland (Figure 5G, Normal 1 and Normal 2 samples). The tumoral samples revealed varying levels of expression of collagenase 3 and pERK1/2. Quantitative analyses of these blots followed by Pearson correlation analysis showed a relationship among the levels of pERK1/2 and collagenase 3 expression (Figure 5h).

Transcriptional control of collagenase 3 by the NRG-ERK1/2 signalling route

The fact that a rise in pERK1/2 preceded the accumulation of collagenase 3 in the culture media suggested that ERK1/2 could be regulating collagenase 3 levels through a transcriptional

mechanism. qPCR experiments were used to analyze whether U0126 could affect the transcription of the mRNA coding for collagenase 3. These experiments demonstrated that ERK1/2 likely controlled the expression of collagenase 3 by acting on its transcription (Figure 6a). Progressive deletion analyses of the human collagenase 3 promoter indicated a region from bases -492 to -453 which contained most of the NRG-responsive sequence of the collagenase 3 promoter (Figure 6b). Bioinformatic analysis of this region indicated potential binding sites for the Pit-1, c-Myb, YY1, NF-1 and H4TF2 transcription factors. RT-PCR analyses detected expression of YY1 mRNAs in MCF7 cells, while mRNAs for the rest of the transcription factors was low or undetectable (Figure 6c). Individual mutation of sites recognized by YY1 in the -492 to -453 region failed to link YY1 to the regulation of collagenase 3 transcription (Figures 6d and e). We further exploited bioinformatics tools in an attempt to define the ERK-responsive region of the collagenase 3 promoter. We detected a sequence which matched with one reported to be recognized by the plant transcription factor SBF1^{29,30} (Figure 6d).

Mutations in this sequence fully prevented NRG-induced upregulation of luciferase activity, pointing to that region as the one responsible for the control of collagenase 3 expression by the NRG-ERK1/2 axis. Together, the composite analyses of these results indicate that NRGs transcriptionally control collagenase 3 expression through activation of the ERK1/2 pathway.

DISCUSSION

ErbB/HER receptors have attracted substantial interest because of their involvement in the pathogenesis of several types of tumors.⁷ Activation of these receptors through interaction with their ligands has been shown to facilitate tumorigenesis in several oncological diseases, including breast cancer.^{31,32} In the latter, we show here that expression of NRGs is linked to poor patient outcome. Moreover, the expression of NRGs was associated to metastatic relapses. This latter conclusion was supported by the statistical association between NRG expression in the primary tumor and tumoral cell invasion of lymph nodes. That NRGs favored metastatic dissemination of breast cancer cells was confirmed using preclinical *in vivo* models. In fact, mice injected with MCF7 cells expressing NRGα2c developed metastatic lesions. This is a relevant finding, because it is well known that MCF7 cells are poorly metastatic. In fact, we were unable to detect metastatic lesions in mice injected with wild-type MCF7 cells. However, it is also important to point out that metastatic dissemination of MCF7-NRGα2c cells was not observed in all the mice injected with those cells, indicating that NRG expression may facilitate dissemination of breast cancer cells only in the presence of optimal *in vivo* conditions.

We reasoned that the NRG-ErbB/HER axis could favor dissemination of tumoral cells to metastatic sites, and that idea moved us to explore mediators of such an effect. Microarray analyses identified changes in transforming growth factor-β signaling, integrins and metalloprotease genes upon activation of NRG receptors in MCF7 cells. qPCR together with western blotting confirmed these changes at the RNA and protein level, and revealed a substantial accumulation of collagenase 3 in cells treated with NRG. This fact, together with the participation of metalloproteinases in the dissemination of tumoral cells,³³ led us to focus on collagenase 3 as a potential important mediator of the prometastatic actions of NRGs. Accumulation of collagenase 3 in the culture media of MCF7 cells resulted in an increase in its activity, which facilitated cell motility and invasion *in vitro*. *In vivo*, collagenase 3 reduction decreased local spreading as well as metastatic dissemination of MCF7-NRGα2c cells to sites distant from the primary place of injection. These facts suggest that the expression of collagenase 3 may not only facilitate distant dissemination of breast cancer cells, but may also favor local spreading of the primary tumoral mass. These results fall in line with former reports which linked collagenase 3 levels to the expression of HER2 and aggressiveness of breast tumors.^{25,26,34–36} Our data also indicated increases in stromelysin 2 expression, and others have reported increases in MMP-9 upon activation of NRG receptors.¹¹ Together, these data suggest that MMP expression is tightly regulated by NRG receptors and is responsible of important biological properties of breast cancer cells, including local spreading and distant dissemination.

Mechanistic studies identified the ERK1/2 signaling axis as the pathway controlling the regulation of collagenase 3 levels upon activation of NRG receptors. This conclusion was supported by the use of pharmacological as well as genetic strategies which acted on the ERK1/2 route. Moreover, statistically significant correlation was found between pERK1/2 and collagenase 3 levels in patient-derived tumor samples. Activation of the ERK1/2 route caused upregulation of the mRNA coding for collagenase 3. Deletion mapping of the collagenase 3 promoter defined the region, located between nucleotides –492 to –455, involved in the

regulation of the expression of collagenase 3 upon activation of NRG receptors. Bioinformatic analyses detected a nucleotide sequence which in plants is recognized by the transcription factor SBF1,³⁰ and whose mutation was able to prevent NRG-induced upregulation of collagenase 3 expression. This finding opens a relevant question of whether SBF1 orthologs exist in mammalian cells.

The description that NRG expression is linked to metastatic dissemination of breast cancer cells together with the identification of collagenase 3 as an important component in such dissemination process may have clinical implications. From the diagnostic and biomarker point of view, we describe a pathway linked to breast cancer dissemination that can be recognized in patients by evaluating NRG expression in their primary tumors. In addition, the definition of components of this pathway offers therapeutic possibilities. In fact, the availability of agents acting on HER receptors⁷ or the ERK route³⁷ raises the possibility of incorporating these agents to the armamentarium to fight breast cancer dissemination, especially in circumstances in which NRG receptors are active. It will be exciting to analyze this possibility in the clinical setting, and also to identify other oncological entities in which the NRG-collagenase 3 signaling axis may have a relevant role in the control of tumor cell dissemination.

MATERIALS AND METHODS

Cell culture and transfections

MCF7, T47D and SKBR3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, in a 95:5 air-CO₂ atmosphere at 37 °C. Transfections of MCF7 cells were performed with JetPEI DNA transfection reagent (Polyplus-transfection SA, Illkirch, France) following the manufacturer's instructions. To knockdown collagenase 3, MCF7 cells were transfected using JetPEI with lentiviral vectors (Thermo Fisher Scientific, Rockford, IL, USA) containing at least four different short hairpin RNA-coding sequences. MCF7 cells expressing proNRGα2c (MCF7-NRGα2c) have been described.¹⁴ To generate MCF7 cell lines that expressed luciferase (MCF7-Luc and MCF7-NRGα2c-Luc), cells were transfected with pCDNA3-Luc, and clones selected with 200 µg/ml of hygromycin. Cultures were incubated with 10 mg/ml of Luciferin Potassium Salt (Regis Technologies, Morton Grove, IL, USA) and luciferase activity of each clone measured in a Gen5 (BioTek, Winooski, VT, USA) apparatus.

Cell proliferation, immunoprecipitation, western blotting and antibody arrays

For cell proliferation analyses, cells were plated at 10 000 cells/well and cultured overnight in Dulbecco's modified Eagle's medium+10% fetal bovine serum. The next day (day 1 of culture) cells were treated, and an MTT assay was performed³⁸ and considered the starting point. Preparation of cell lysates for immunoprecipitation or western analyses was as described.^{39,40} For the antibody arrays, 600 µg of MCF7 cell lysates treated or not with NRG were hybridized to ARY003B antibody arrays (R&D Systems, Abingdon, UK). Quantitation of pixel densities of the different spots corresponding to 43 signalling mediators was performed using the Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Collagenase 3 activity studies and collagenase 3 promoter analyses

Total active collagenase 3 in the culture medium was evaluated using a SensoLyte 520 collagenase 3 Fluorimetric Assay Kit (AnaSpec, Fremont, CA, USA). To perform luciferase assays, MCF7 cells were seeded in 60-mm plates and were transfected with 1 µg of each plasmid containing the different fragments of the collagenase 3 human promoter. Forty-eight hours later, the cells were treated with NRG during 3 h and the luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and measured in a Gen5 apparatus. Collagenase 3 luciferase levels were normalized with respect to the renilla signal (50 ng of pCDNA3-Renilla transfected per well) used as transfection control.

Microarray RNA analyses

Total RNA was extracted and purified with the PureLink Micro-to-Midi kit (Invitrogen, Paisley, UK). Double-stranded cDNA and afterwards biotinylated cRNA were synthesized using a T7-polyT primer and the BioArray RNA labeling kit (Enzo, Farmingdale, NY, USA), respectively. The labeled RNA was then fragmented and hybridized to Human Gene 1.0 ST oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). For the microarray data analysis, the CEL files from each microarray hybridization (three for each condition, prepared from three different experiments) were imported into the dChip software.⁴¹ A twofold change was used as the threshold to identify genes whose expression was modified (upregulated or downregulated) by the treatment with NRG. Functional categorization of the genes was performed with the Ingenuity software.

Xenograft studies

Mice were manipulated at the animal facility of the University of Salamanca following legal and institutional guidelines. Female BALBc *nu/nu* mice (7 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). MCF7-Luc, MCF7-NRGa2c-Luc and MCF7-NRGa2c-shcollagenase 3-Luc cells (5×10^6) in 100 μ l of Dulbecco's modified Eagle's medium+10% fetal bovine serum and 100 μ l of Matrigel were injected into the mammary fat pad. Anesthetized mice were intraperitoneally injected with 100 mg/kg Luciferin Potassium Salt and bioluminescence images were acquired with an IVIS 50 Imaging System (Xenogen, Alameda, CA, USA). Analyses were performed with the Living Image software (Xenogen) by measuring photon flux.

Tumor samples obtained after killing the animals were immediately frozen in liquid nitrogen. The tumors were minced, washed with phosphate-buffered saline and homogenized (Dispomix, L&M Biotech, Holly Springs, NC, USA) in ice-cold lysis buffer (1.5 ml/100 mg tumor). This homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C, and the supernatants were transferred to new tubes. The expression of collagenase 3 was analyzed by western blotting.

Patient samples

Collagenase 3, NRG, ERK1/2 and pERK1/2 expression was evaluated by western blotting in breast cancer samples from patients of the University Hospital of Salamanca. The frozen samples were randomly selected from patients who had undergone surgery through the years 2004–2008 and from whom frozen tissue was available. Protein concentration was measured by the Bradford assay, and 2 mg were immunoprecipitated with the anti-collagenase 3 antibody, or 50 μ g of protein loaded directly in a gel to measure pERK1/2 and ERK1/2.

Immunohistochemistry

Five-micrometer sections were cut with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slides. Immunohistochemistry was carried out on these sections using a TechMate TM50 autostainer (Dako, Glostrup, Denmark). Tissue sections were deparaffinized in xylene, and then rehydrated in decreasing concentrations of ethanol (100, 96, 80, 70%, then water). To enhance antigen retrieval, sections were microwave-treated (H2800 Microwave Processor, EBS Sciences, East Granby, CT, USA) in citrate buffer (Target Retrieval Solution, Dako) at 99 °C for 1 min. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxylin, dehydrated with ethanol and permanently coverslipped. The dilution of the anti-proNRG or the anti-collagenase 3 antibodies was 1:50. Details of the scoring method have previously been reported.¹⁷

Statistical analyses

Comparison of continuous variables between two groups for *in vitro* assays and xenograft mice experiments were made using a two-tailed Student's *t*-test. Results were considered to be statistically significant when *P* values were below 0.05. Statistical data are presented as the mean \pm standard deviation or 95% confidence intervals. The number of experiments performed is indicated in the corresponding figure legend. DFS was calculated from the date of diagnosis to the date of recurrence or death. Kaplan-Meier survival analyses were carried out for DFS. Differences in DFS according to NRG expression were compared using the Log-rank test. Multivariate analysis using the Cox proportional hazards models was

performed to define prognostic independent factors for DFS. All statistical tests were conducted at a two-sided significance level of 0.05, and were performed using the statistical software SPSS 15.0 (SPSS Inc, Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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