

## Insulin-like Growth Factor– Dependent Proliferation and Survival of Triple-Negative Breast Cancer Cells: Implications for Therapy<sup>1</sup>

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### Abstract

Triple-negative breast cancers have a poor prognosis and are not amenable to endocrine- or HER2-targeted therapies. The prevailing view is that targeting the insulin-like growth factor (IGF) signal transduction pathway will not be beneficial for triple-negative breast cancers because their growth is not IGF-responsive. The present study investigates the importance of IGFs in the proliferation and survival of triple-negative breast cancer cells. Estrogen and progesterone receptors, HER2, type I IGF, and insulin receptors were measured by Western transfer analysis. The effects of IGF-1 on proliferation were assessed by DNA quantitation and on cell survival by poly (ADP-ribose) polymerase cleavage. The effect of IGF-1 on phosphorylation of the IGF receptors, Akt and mitogen-activated protein kinase, was measured by Western transfer analysis. Seven cell lines were identified as models of triple-negative breast cancer and shown to express IGF receptors at levels similar to those present in estrogen-responsive cell lines known to respond to IGFs. IGF-1 increased the proliferation and cell survival of all triple-negative cell lines. Proliferation was attenuated after reduction of type I IGF receptor expression. Cells that express higher levels of receptor were more sensitive to subnanomolar IGF-1 concentrations, but the magnitude of the effects was not correlated simply with the absolute amount or phosphorylation of the IGF receptors, Akt or mitogen-activated protein kinase. These results show that IGFs stimulate cell proliferation and promote cell survival in triple-negative breast cancer cells and warrant investigation of the IGF signal transduction pathway as a therapeutic target for the treatment of triple-negative breast cancer.

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### Introduction

Breast cancer is the most common female cancer in the western world. Despite improvements in early diagnosis and in clinical management, breast cancer kills more than 520,000 people worldwide each year. Approximately 15% of breast cancers are classified as triple-negative because they do not express estrogen receptor, progesterone receptor, or HER2 [1,2]. There is considerable overlap between triple-negative tumors, basal-like tumors, and tumors, which expresses cytokeratins 5 and 6 [3]. Triple-negative tumors are more prevalent in younger [4] and in black women [4,5]. They account for 27% of breast cancer cases diagnosed in premenopausal African Americans, 25% in younger black British women, and 27% in all indigenous Africans [6,7]. Basal-like breast cancers have a tendency toward visceral, *versus* bone, metastasis [7,8]. They present at a similar clinical stage to other subtypes but

have a worse prognosis [8]. BRCA1, but not BRCA2-associated, breast tumors are predominantly triple-negative or basal-like [9,10].

Women with triple-negative tumors have limited therapeutic options. They are unlikely to benefit from endocrine therapies that exploit the dependence of tumor cells on estrogens or from trastuzumab, the anti-HER2 antibody, which targets the dependence of tumor cells on

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signaling through the HER2 oncogene. They are treated with conventional chemotherapies, which have limited efficacy and unpleasant adverse side effect profiles [1,11,12]. There is an urgent need for non-cytotoxic, targeted therapies that could prolong the lives of women with triple-negative breast cancer.

Insulin-like growth factors (IGFs) of which there are three ligands, namely, IGF-1, IGF-2, and insulin, transmit their signals through two paralogous receptor proteins located in the plasma membrane: the type I IGF receptor and the insulin receptor (IGF receptors). The type I IGF and insulin receptors are heterotetrameric proteins with intrinsic phosphotyrosine activity. IGF-1 and IGF-2 have higher affinities for the type I receptor, whereas insulin has a relatively higher affinity for the insulin receptor. The ligands interact with extracellular domains of the receptors and induce phosphorylation of and conformational changes in the receptors, which, in turn, facilitate recruitment and phosphorylation of the intracellular adaptor proteins. The signaling cascades initiated lead to activation of proteins such as mitogen-activated protein kinase (MAPK) and Akt and ultimately to increased cell survival, proliferation, and migration [13].

The IGF system is involved in tumorigenesis and the proliferation, survival, and migration of tumor cells. High circulating IGF-1 concentrations and low blood IGF binding protein concentrations are a risk factor for several types of cancer including breast cancer [14–16]. Components of the IGF signal transduction pathway are expressed widely and often at high levels in cancer cells [17].

IGF antagonists have been developed, several of which have entered clinical trials [18–22]. These include humanized antibodies to the type I IGF receptor and tyrosine kinase inhibitors. In breast cancer, the antibody figitumumab is being tested in combination with exemestane and docetaxol, the tyrosine kinase inhibitor AMG479 in combination with exemestane and fulvestrant, and the antibody cixutumumab with temsirolimus and lapatinib or capecitabine in HER2-positive breast cancers.

The emphasis of the breast cancer trials has been to try to augment the effect of hormone therapy, the rationale being based on laboratory studies that show that there are synergistic effects of estrogens and IGFs on the proliferation of estrogen-responsive breast cancer cells [23–27]. The presence of a functional IGF signal transduction pathway in estrogen-unresponsive breast cells has received little attention and is controversial. One study reported that estrogen-unresponsive cells show proliferative response to IGFs [23], but most studies conclude that estrogen unresponsive cells show no mitogenic response to IGFs [28–31].

In the present study, we reevaluate the importance of IGF signal transduction in triple-negative breast cancer cells. We show that all triple-negative breast cancer cells tested express IGF receptors, that components of the IGF signal transduction pathway are phosphorylated in response to IGF-1, and that IGF-1 stimulates cell proliferation and cell survival. This preclinical study provides a rationale for the future clinical investigation of the therapeutic efficacy of type I IGF receptor inhibitors in the treatment of triple-negative breast cancer.

## Materials and Methods

### Cell Culture

Breast cell lines established from malignant breast epithelial cells: BT-474, MCF-7, T-47D, ZR-75, EFF-3, EFM-19, MDA-MB-435s, Hs 578T, BT-20, and SK-BR-3 cells were cultured routinely in Dulbecco modified Eagle medium (DMEM), supplemented with 10%

fetal calf serum (FCS) and 1  $\mu\text{g}/\text{ml}$  insulin. MDA-MB-231 cells were cultured in DMEM, 5% FCS, and 1  $\mu\text{g}/\text{ml}$  insulin; MDA-MB-468 cells were cultured in DMEM/Hams F12 nutrient, 10% FCS, and 1  $\mu\text{g}/\text{ml}$  insulin; and BT-549 cells were cultured in RPMI, 10% FCS, and 1  $\mu\text{g}/\text{ml}$  insulin. HBL-100 breast epithelial cells that were isolated from human milk but have become transformed during culture were cultured in DMEM, 10% FCS, and 1  $\mu\text{g}/\text{ml}$  insulin. Spontaneously immortalized MCF-10A breast epithelial cells isolated from normal breast tissue were cultured in DMEM/Hams F12 nutrient mix supplemented with 5% horse serum, 0.5  $\mu\text{g}/\text{ml}$  hydrocortisone, 100 ng/ml cholera toxin, 10  $\mu\text{g}/\text{ml}$  insulin, and 20 ng/ml EGF. Cells were obtained from the American Type Culture Collection (Manassas, VA). It was suggested that the MDA-MB-435s cell line might be of melanoma origin, but it has been validated as a breast cancer cell line [32,33]. Nonimmortalized human mammary epithelial cells (HUMECs) were cultured in the specialist medium provided by the supplier (Invitrogen, Paisley, United Kingdom).

### Western Transfer Analysis

Cells were grown in routine culture media and lysed in RIPA lysis buffer, and the protein concentration was measured with a bicinchoninic acid assay (Thermo Scientific, Loughborough, UK). Equal amounts of protein were separated by polyacrylamide gel electrophoresis and transferred to 0.45- $\mu\text{m}$  nitrocellulose membrane [34,35]. Membranes were incubated with specific antibodies: estrogen receptor  $\alpha$  (05-820; Upstate Biotechnology, Inc, Watford, United Kingdom); HER2 (NCL-CBE1; Novocastra, Milton Keynes, United Kingdom); type I IGF receptor (#3027), phospho-IGF receptors (#3024) phospho-ERK1/2 (#9102), and phospho-Akt (#4060) (Cell Signaling Technologies, Hitchin, United Kingdom); insulin receptor (sc-81466) and GAPDH (Santa Cruz Biotechnology, Heidelberg, Germany);  $\alpha$ -tubulin (Sigma, Poole, United Kingdom); and poly (ADP-ribose) polymerase (PARP, 556494; BD Biosciences, Oxford, United Kingdom) using the concentrations and conditions recommended by the supplier. Phospho-specific antibodies that detect activated type I IGF receptor autophosphorylated on tyrosines 1135 and 1136 interact also with activated insulin receptor autophosphorylated on tyrosines 1150 and 1151. The phospho-MAPK antibody detects Erk1 when it is phosphorylated on Thr 202 and Tyr 204 and Erk2 when it is phosphorylated on Thr 185 and Tyr 187. The phospho-Akt antibody detects Akt when it is phosphorylated on Ser 473. Proteins were visualized by incubating the filters with horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence with SuperSignal West Dura Substrate (Thermo Scientific) and exposure to an x-ray film. The intensity of the signal was quantified by densitometric analysis using LabWorks 4.0 software (UVP, Inc, Cambridge, United Kingdom).

### Cell Proliferation

Cells were seeded into 16-mm-diameter wells at a density of 4000 cells per well (MDA-MB-231, MDA-MB-435s), 7000 cells per well (MDA-MB-468, Hs 578T), or 10,000 cells per well (BT-549) allowed to attach for 24 or 40 hours and withdrawn from the effects of untreated serum for 2 to 3 days by replacing the medium twice daily [23,24,36] with growth factor-depleted medium comprising phenol red-free DMEM supplemented with 5% or 10% charcoal-treated calf serum [37]. Cells were incubated subsequently in various concentrations of IGF-1 for 3 to 12 days. Medium was changed daily. Cell growth was determined by measuring the amount of DNA in each well using PicoGreen dsDNA Quantitation Reagent (Invitrogen) [38].

### Knockdown of Type I IGF Receptor

Cells were seeded into 16-mm-diameter wells at a density of 5000 cells per well (MDA-MB-231) or 7000 cells per well (Hs 578T) allowed to attach for 24 hours and grown for 4 days in routine maintenance medium. The medium was changed daily. Cells were washed twice with PBS, maintenance medium was replenished, and cells were transfected with 12.5  $\mu$ g/ml empty vector or shRNA plasmid pKD-IGF-IR-v2 (Upstate Biotechnology) in the presence of a 3:1 ratio of FuGENE HD transfection reagent (Roche, Burgess Hill, United Kingdom) and incubated for 24 hours at 37°C as described previously [35]. Cells were then washed twice with PBS and incubated in withdrawal medium in the absence and presence of 50 ng/ml IGF-1 for 3 days at 37°C. Cells were lysed, and aliquots of protein lysate were analyzed by Western transfer for expression of the type I IGF receptor.

To test the effects of the knockdown on cell proliferation, cells were seeded into 16-mm-diameter wells at a density of 5000 cells per well (MDA-MB-231) or 7000 cells per well (Hs 578T), allowed to attach for 46 hours, washed with PBS, and transfected with the plasmids at a 3:1 ratio of FuGENE HD to DNA. After 18 to 24 hours, the cells were washed twice with PBS, and the medium was replaced with withdrawal medium in the absence and presence of IGF-1 for 3 to 6 days. After 3 days of culture, the cells were retransfected with the plasmids. The medium was changed daily. Cell growth was determined by measuring the amount of DNA in each well using PicoGreen dsDNA Quantitation Reagent (Invitrogen) [38].

### Cell Survival

Cells were seeded into 35-mm-diameter wells at a density of 400,000 cells per well and allowed to attach for 24 hours. Cells were then withdrawn for 2 days and subsequently pretreated with various concentrations of IGF-1 for 1 hour before incubation with 1  $\mu$ M staurosporine for different lengths of time followed by lysis in RIPA buffer. Programmed cell death was evaluated by the extent of PARP cleavage determined by Western transfer analysis with a PARP antibody specific to both cleaved and uncleaved PARP as described previously.

### IGF-I–Stimulated Protein Phosphorylation

Cells were seeded into 35-mm-diameter wells in routine culture medium and allowed to attach for 24 hours. Cells were then withdrawn from the stimulating effects of untreated serum for 2 days. The medium was changed twice daily with phenol red–free DMEM supplemented with 10% dextran-coated charcoal-treated calf serum [37]. Cells were then incubated for 2 hours in serum-free medium and subsequently in various concentrations of IGF-1 for 15 minutes and lysed in RIPA buffer. Phosphorylated proteins were detected by Western transfer analysis as described above.

### Statistics

Experiments were replicated at least three times. For the results shown in Figures 1–5, a representative example is shown. For the results shown in Figures 3B, 6, and 7, data were normalized and expressed as a percentage of the maximum IGF-stimulated effect. Results from independent experiments are expressed as means  $\pm$  SEM. Differences between groups were tested by analysis of variance, paired or unpaired *t* test; *P* < .05 was considered statistically significant.

## Results

### Receptor Expression

Expression of the estrogen receptor, progesterone receptor, and HER2 was assessed in 14 transformed and 2 untransformed human breast epithelial cell lines (Figure 1). There was variable but generally high expression of estrogen receptor in the six known estrogen-responsive cell lines, BT-474, MCF-7, T-47D, ZR-75, EFF-3, and EFM-19 [39–41]. EFM-19 cells express the highest and BT-474 cells the lowest levels (*t* test, *P* < .05). No estrogen receptor was detected in the other cell lines even after prolonged exposure of the filters to the x-ray film.

Expression of the progesterone receptor was restricted also to the estrogen-responsive cell lines [42]. Expression was relatively high in MCF-7, T-47D, and EFM-19 cells and was lower in BT-474, ZR-75, and EFF-3 cells (*t* test, *P* < .05). HER2 was expressed at highest levels in SK-BR-3 and BT-474 cells (*t* test, *P* < .05), in which HER2 is amplified [39], in MCF-7, T-47D, EFF-3, and EFM-19 breast cancer cells and in MCF-10A and HUMEK untransformed breast cells. These results show that seven of the cell lines, BT-20, MDA-MB-231, MDA-MB-468, MDA-MB-435s, Hs 578T, BT-549, and HBL-100, do not express estrogen and progesterone receptors or HER2 and are potential models of triple-negative breast cancer.

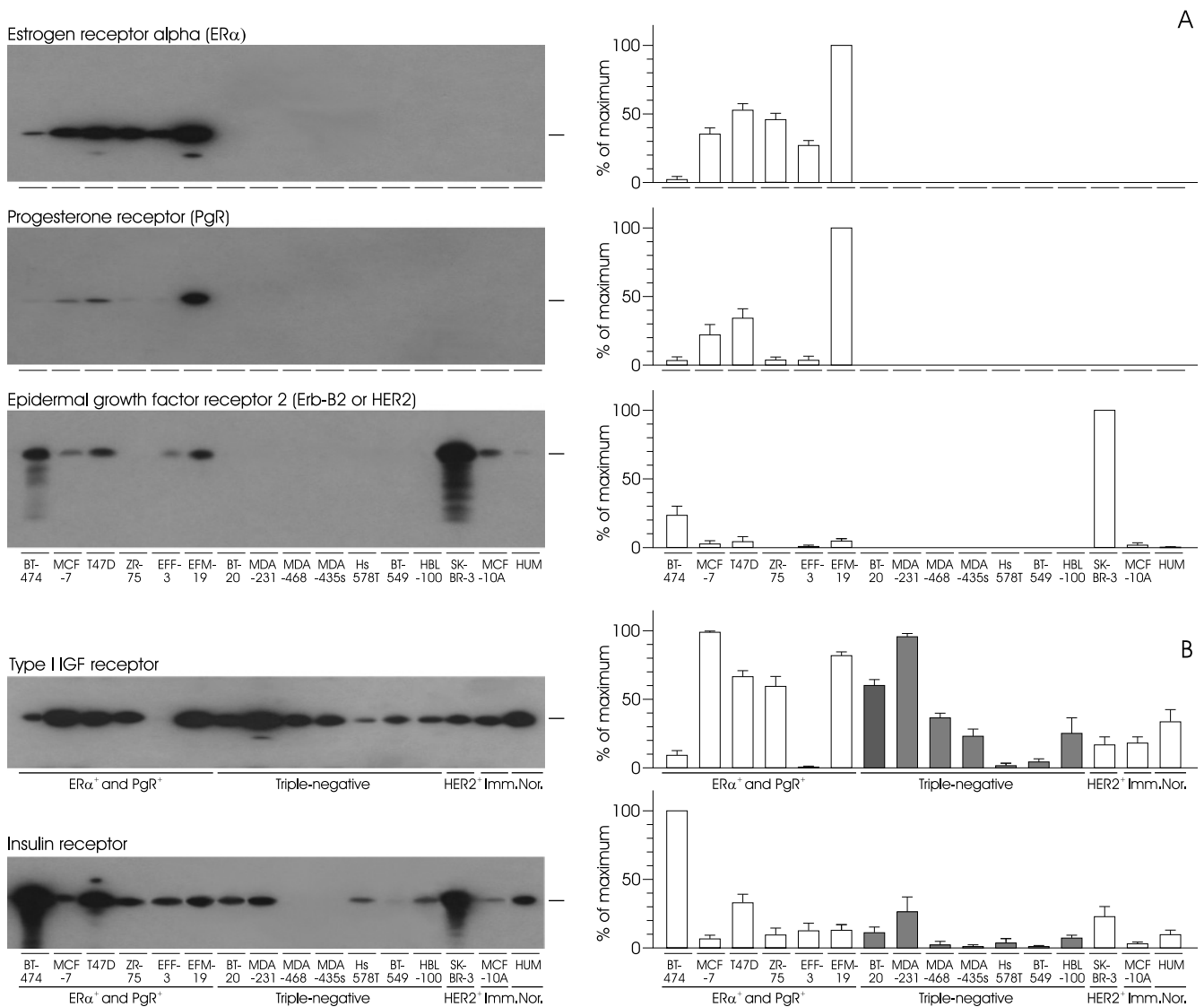
To evaluate if IGF signal transduction pathways might be important in triple-negative breast cancer cells, the expression of the type I IGF and insulin receptors was compared with expression in estrogen-responsive cells that are known to respond to IGFs (Figure 1B). All cell lines expressed the type I IGF receptor. Among the estrogen-responsive cell lines, MCF-7 and EFM-19 cells express the highest and EFF-3 the lowest levels (*t* test, *P* < .05). Of the triple-negative breast cancer cell lines, BT-20, MDA-MB-231, MDA-MB-468, MDA-MB-435s, and HBL-100 express high levels similar to those in MCF-7 and EFM-19 cells. The triple-negative cell lines that express lower levels of the type I IGF receptor still express more receptor than EFF-3 cells (*t* test, *P* < .05). The SK-BR-3 and untransformed MCF-10A cell lines express moderate amounts of the receptor, whereas HUMEK normal breast epithelial cells express higher levels.

The insulin receptor was detected in all estrogen-responsive and triple-negative cell lines. The highest expression was in BT-474, T-47D, and SK-BR-3 (*t* test, *P* < .05), whereas it was lower in MDA-MB-468, MDA-MB-435s, and BT-549 cells. The ratio of type I IGF to insulin receptors varied across the 16 cell lines. MCF-7, EFM-19, MDA-MB-231, MDA-MB-435s, and BT-549 cells expressed relatively higher levels of the type I IGF receptor, whereas BT-474, EFF-3, and SK-BR-3 cells expressed relatively higher levels of the insulin receptor. T-47D cells expressed high levels of both receptors and Hs 578T cells expressed low levels of both receptors.

These results show considerable variation in expression of the IGF receptors but overall expression is similar in triple-negative and estrogen-responsive cells. The relatively high expression of the IGF receptors in triple-negative breast cancer cells suggests that the IGF signal transduction pathway may be important in controlling cell proliferation and cell survival in this subtype of breast cancer.

### Effect of IGF-1 on Triple-Negative Breast Cancer Cell Proliferation and Survival

Although screens with small-molecule inhibitors of the type I IGF receptor have reported efficacy in triple-negative breast cancer cell lines [21], and we have reported previously that IGF-1 stimulates the proliferation of triple-negative MDA-MB-231 cells [23], the



**Figure 1.** Expression of steroid and growth hormone receptors in cultured breast cells. BT-474, MCF-7, T-47D, ZR-75, EFF-3, EFM-19, BT-20, MDA-MB-231 (MDA-231), MDA-MB-468 (MDA-468), MDA-MB-435s (MDA-435s), Hs 578T, BT-549, HBL-100, SK-BR-3, MCF-10A, and HUMEC (HUM) cells were grown to 70% confluence in routine culture medium and lysed, and 20  $\mu$ g of protein was electrophoresed on polyacrylamide gels and transferred to nitrocellulose as described in the Materials and Methods. Membranes were incubated with estrogen receptor (67 kDa), progesterone receptor (118 kDa), HER2 (185 kDa) (A), type I IGF receptor (95 kDa), or insulin receptor (95 kDa) (B) antibodies and developed by enhanced chemiluminescence as described in the Materials and Methods. Representative Western transfer images are shown on the left-hand side of the figure. The membranes were incubated secondly with a GAPDH antibody (data not shown), which confirmed equal loading and transfer. The analysis was repeated at least twice on four or more individual samples prepared from MCF-7, T-47D, ZR-75, EFF-3, EFM-19, MDA-MB-231, Hs 578T, BT-20, HBL-100, and SK-BR-3 breast epithelial cells and on three individual samples prepared from BT-474, MDA-MB-435s, MDA-MB-468, BT-549, MCF-10A, and HUMEC cells. The amount of each protein was determined by densitometric scanning of x-ray films followed by analysis with LabWorks 4.0 software and correcting for GAPDH as described in the Materials and Methods. The amount of each protein was expressed as the percentage of the amount of protein in the cell line that expresses the most of that protein and is represented in the histograms shown on the right hand side of the figure. Mean values  $\pm$  SEM are shown. The filled columns indicate expression in triple-negative cell lines.

current view is that IGFs are not mitogenic for triple-negative breast cancer cells [28–31]. After demonstration of significant expression of IGF receptors by triple-negative breast cancer cell lines, we investigated the effect of IGF-1 on their growth. Growth rates of MDA-MB-231, MDA-MB-435s, Hs 578T, and BT-549 cells were similar in the absence of IGF-1 (Figure 2). IGF-1 stimulated the proliferation of these four cell lines. MDA-MB-231 cells were the most responsive to IGF-1 treatment and cell numbers were increased eight-fold after

9 days of treatment. MDA-MB-435s and Hs 578T showed intermediate responses with cell numbers increased approximately four-fold, whereas BT-549 cells were less responsive and were increased two-fold.

MDA-MB-468 cell proliferation was stimulated by IGF-1, but the cells were rounded and attached poorly to standard tissue culture plastic. Corning CellBIND plates (Fisher Scientific, Loughborough, United Kingdom) improved cell attachment. MDA-MB-468 cells

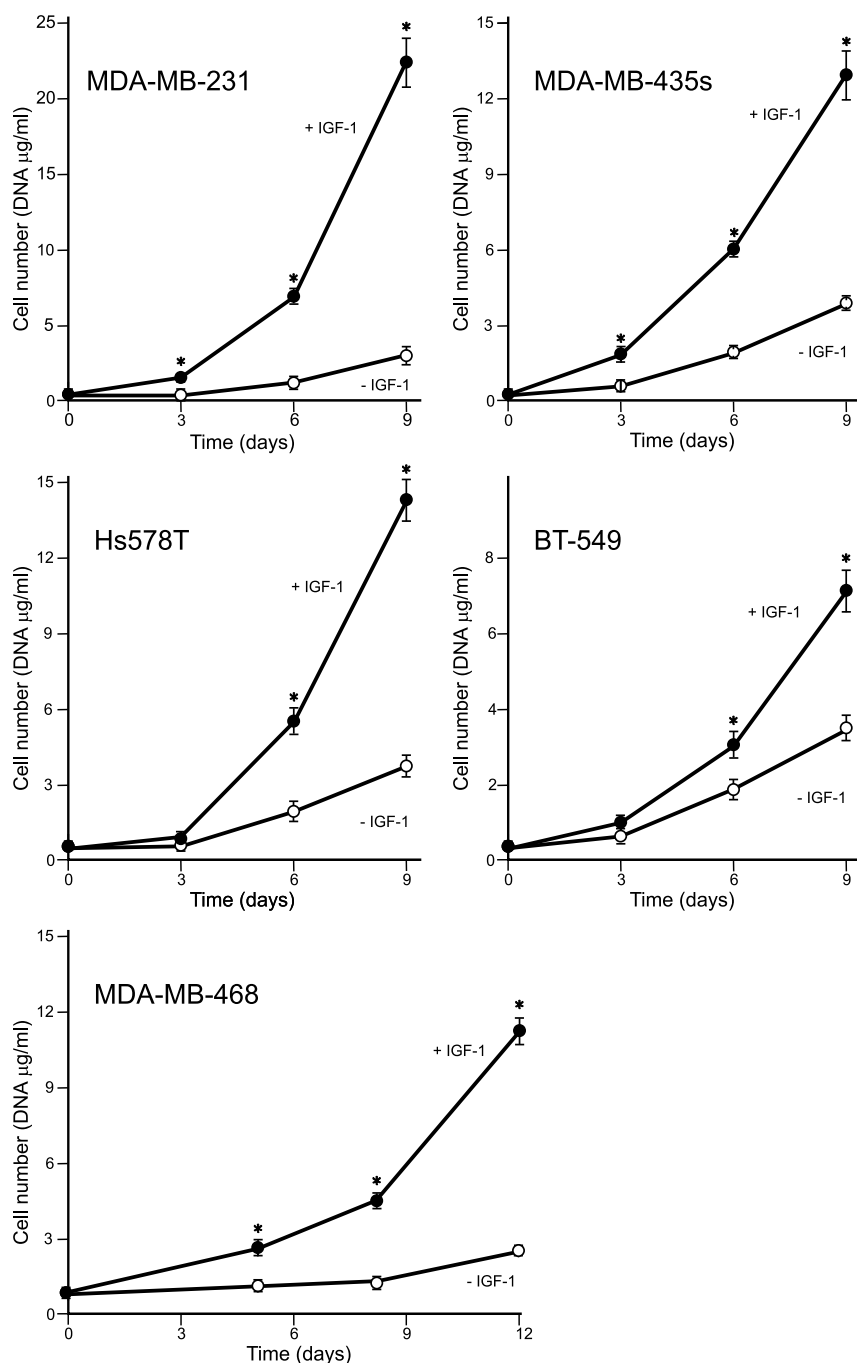
grew more slowly in the growth factor–depleted medium than the other triple-negative breast cancer cells; however, cell numbers were increased four-fold after 12 days of treatment with IGF-1.

In contrast to most studies, our experiments establish unequivocally that cell growth of triple-negative breast cancer cells is increased by IGF-1.

The effect of decreasing the expression of the type I IGF receptor on IGF-1–stimulated cell proliferation was tested. The expression of type I IGF receptor by MDA-MB-231 and Hs 578T cells was reduced to

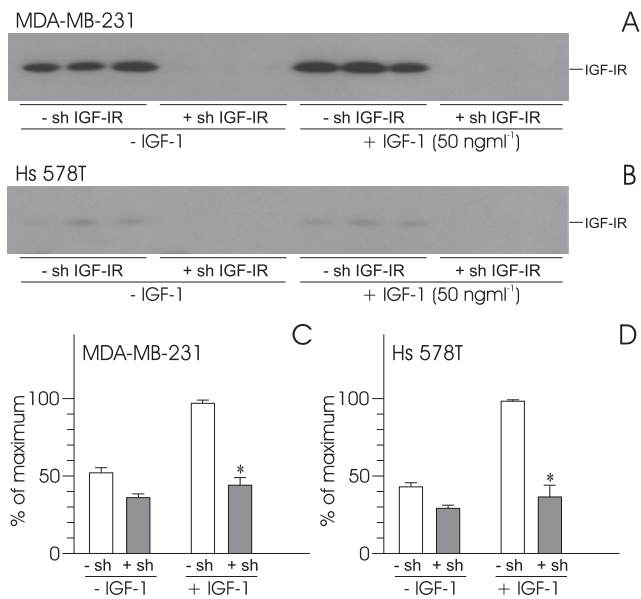
barely detectable levels after transfection of the cells with an shRNA plasmid as described in the Materials and Methods (Figure 3, *A* and *B*). The proliferative response of MDA-MB-231 and Hs 578T cells was reduced significantly in cells in which endogenous expression of the type I IGF receptor had been lowered (Figure 3, *C* and *D*).

IGFs protect a wide variety of cell types against cell death induced by insults including ionizing radiation and cytotoxic drugs both of which are used widely in the treatment of cancer. There have been



**Figure 2.** Stimulation of proliferation of triple-negative breast cancer cells by IGF-1. MDA-MB-231, MDA-MB-435s, Hs 578T, and BT-549 cells were plated in 16-mm-diameter tissue culture wells, allowed to attach, withdrawn from the growth stimulatory effects of normal culture medium, and then cultured for the indicated lengths of time in the absence (○) or presence (●) of 50 ng/ml IGF-1 as described in the Materials and Methods. MDA-MB-468 cells were plated in Corning CellBIND plates. Cells were lysed, and the DNA content of the wells was measured. Bars, SEM. Asterisks show times at which there were significantly more cells in the presence of IGF-1 than in its absence.





**Figure 3.** Effect of decreased expression of type I IGF receptor on cell proliferation in MDA-MB-231 and Hs 578T cells. MDA-MB-231 (A) and Hs 578T (B) cells were transfected with shRNA plasmid pKD-IGF-IR-v2 and then cultured for 3 days in the absence or presence of 50 ng/ml IGF-1. Cells were lysed, and 20  $\mu$ g of protein was electrophoresed on polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated with type I IGF receptor (95 kDa) antibody and developed by enhanced chemiluminescence as described in the Materials and Methods. Representative Western transfer images after exposure to the x-ray film for 30 seconds (A) and 3 hours (B) are shown. The membranes were incubated secondly with a GAPDH antibody (data not shown), which confirmed equal loading and transfer. MDA-MB-231 (C) and Hs 578T (D) cells were transfected with shRNA plasmid pKD-IGF-IR-v2 and then cultured in the absence or presence of 50 ng/ml IGF-1 as described in the Materials and Methods. Cells were lysed, and the DNA content of the wells was measured. Bars, SEM. Asterisks show times at which there were significantly fewer cells grown in the presence of 50 ng/ml IGF-1 in the wells that contained cells transfected with pKD-IGF-IR-v2 than in wells that contained cells transfected with the control plasmid (*t* test,  $P < 0.05$ ).

no studies of the cell survival effect of IGFs in triple-negative breast cancer. Programmed cell death through the caspase-dependent pathway leads to cleavage of PARP from 113 to 89 kDa, thus removing the DNA binding domain. There was no detectable PARP cleavage in any of the cell lines after culture in growth factor-depleted medium. Therefore, to investigate whether IGF-1 promotes cell survival in triple-negative breast cancer cells, the ability of IGF-1 to inhibit staurosporine-induced cell death was measured. Staurosporine binds with high affinity to the ATP binding site of protein kinases, which inhibits their enzymatic activity and activates the caspase-dependent apoptotic pathway.

The triple-negative cells responded differently to staurosporine. Typically, PARP cleavage after the addition of staurosporine was observed first after 4 hours (MDA-MB-231 and HBL-100) but BT-549 cells responded more slowly (Figure 4A). The degree of protection against staurosporine conferred by IGF-1 varied but was generally pronounced; the time taken for cleaved PARP to become detectable increased and the percentage of cleaved PARP at later times decreased in the presence of IGF-1. Exposure of MDA-MB-468 cells to staurosporine for 2 and 4 hours decreased the amount of 113-kDa uncleaved

PARP, and the decrease was attenuated by IGF-1 but cleaved 89-kDa PARP protein was not detected. This suggests that once cleaved by caspases, PARP is degraded rapidly in these cells.

The percentage of cleaved PARP was quantified for the six cell lines in which it was detected (Figure 4B). In Hs 578T cells, the most staurosporine-sensitive cell line, IGF-1 delayed the appearance of cleaved PARP by 30 minutes, and there was a 40% reduction in the amount of cleaved PARP after 2 hours. The delay was longer, and the reduction was greater in the other cell lines. PARP cleavage was delayed by 4 hours in MDA-MB-231 and HBL-100 cells and reduced 20-fold and 10-fold, respectively. It was delayed by 2 hours in BT-20 and MDA-MB-435s cells, in which it was reduced five- and three-fold, respectively. There was no cleaved PARP in the presence of IGF-1 in BT-549 cells.

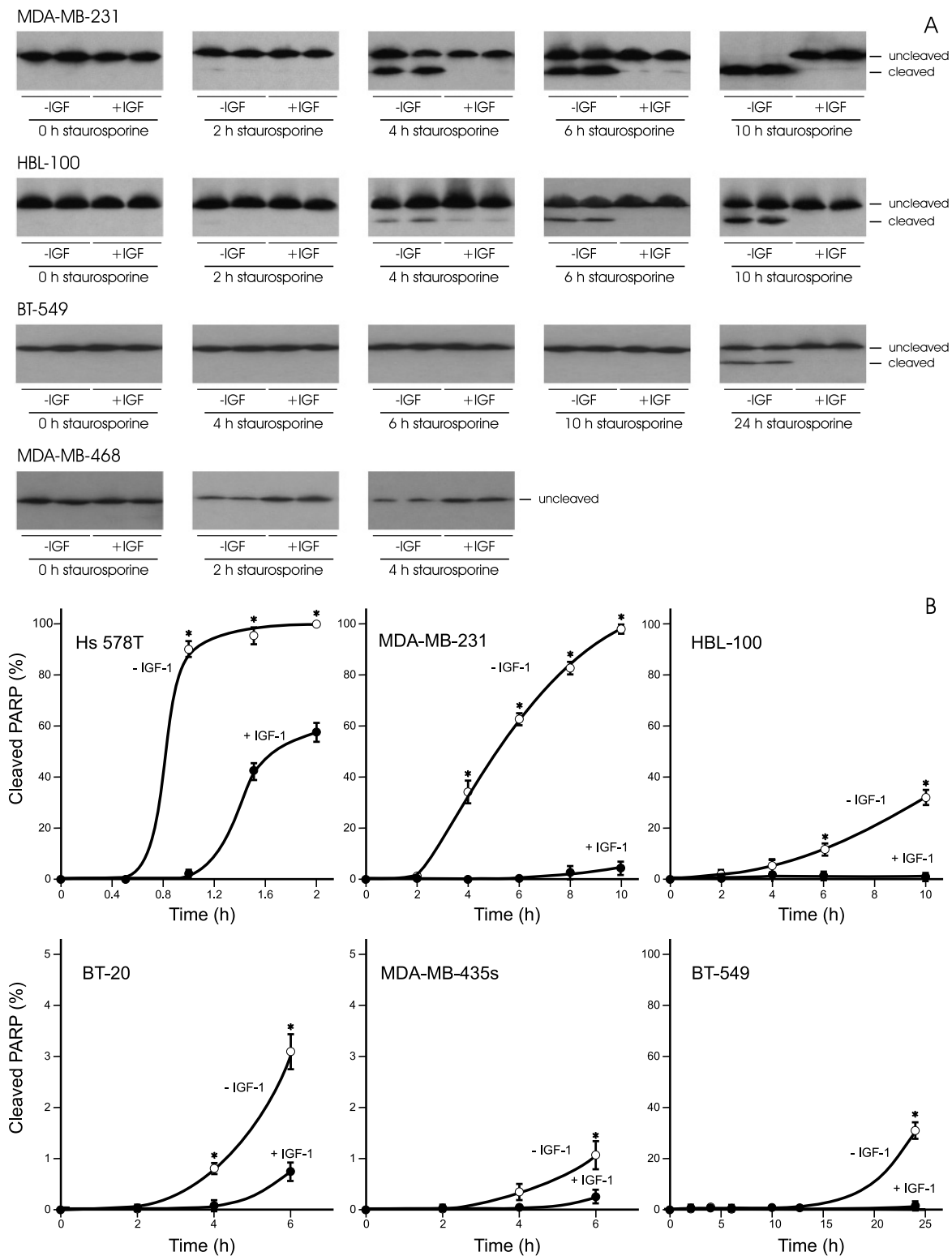
Clearly, these experiments show that IGF-1 has a significant and, in some cases, a dramatic protective effect against staurosporine-induced programmed cell death in triple-negative breast cancer cells. In addition, they provide evidence that the effect of IGF-1 on cell proliferation results from increased cell division rather than decreased cell death because there is no detectable cleaved PARP in any of the cell lines in the absence of staurosporine.

### *IGF-1–Stimulated Phosphorylation of IGF Receptors and Downstream Signal Transduction Proteins*

In general, the level of expression of the type I IGF receptor was related to the response of the cells; for instance, MDA-MB-231 cells that express high levels of receptor show the greatest effects of IGF-1 on cell proliferation and survival. However, there were some anomalies; Hs 578T cells express low levels of type 1 IGF receptor, but both proliferation and survival show large responses to IGF-1. To determine whether this anomaly could involve significant differences in receptor phosphorylation or compensation by other components of the signal transduction pathway, the amount of the phosphorylated IGF receptors and two key downstream signal transduction molecules Akt and MAPK was assessed. Akt functions as a cardinal nodal point for transducing extracellular and intracellular oncogenic signals. MAPK (ERK1/2) is activated preferentially in response to growth factors and phorbol ester and regulates cell proliferation and differentiation.

IGF-1 stimulated phosphorylation of the IGF receptors in all seven triple-negative breast cancer cell lines and in the HER2-positive SK-BR-3 cell line (Figure 5). There was a good correlation between the amounts of receptors expressed and the amount of phosphorylated receptors after treatment with IGF-1. For example BT-20, MDA-MB-231, and HBL-100 cells express high levels of IGF receptors and high levels of phosphorylated receptor were detected after IGF-1 treatment, whereas for Hs 578T cells that contain low levels of receptors, the phosphorylated receptor was visible only after prolonged exposure of the membrane to an x-ray film.

IGF-1 treatment caused pronounced stimulation of the phosphorylation of Akt and of MAPK in the seven triple-negative cell lines and in SK-BR-3. The amount of total Akt and MAPK did not change. The increase in the level of phosphorylated MAPK and Akt after IGF-1 treatment did not always reflect the level of phosphorylated IGF receptor. MDA-MB-231 cells, which have among the highest levels of phosphorylated receptor, have the lowest level of phosphorylated Akt, and MDA-MB-468, Hs 578T and, BT-549 cells, which have low levels of phosphorylated receptor, have relatively high levels of phosphorylated Akt after IGF-1 stimulation. These apparent discrepancies may be explained in part by the total levels of the effector



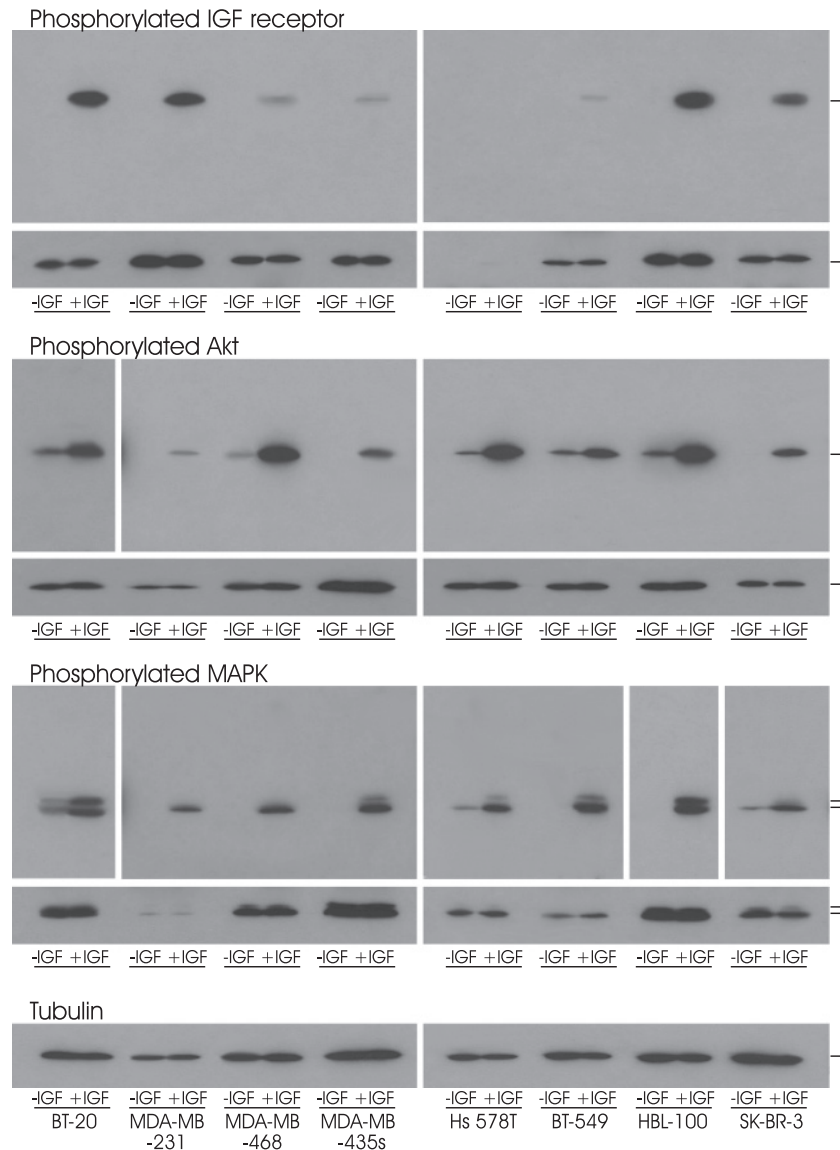
**Figure 4.** Protective effect of IGF-1 on programmed cell death in triple-negative breast cancer cells. MDA-MB-231, HBL-100, BT-549, MDA-MB-468, Hs 578T, BT-20, and MDA-MB-435s cells were cultured and treated with staurosporine in the absence and presence of 50 ng/ml IGF-1 as described in the Materials and Methods. Cells were lysed after various times, and the amount of PARP cleavage was measured by Western transfer analysis. Representative Western transfer images are shown in A. The positions of cleaved and uncleaved PARP proteins are indicated on the right of the images. The amount of each protein was determined by densitometric scanning of the x-ray films followed by analysis with LabWorks 4.0 software. In B, the amount of cleaved 89-kDa form of PARP protein expressed as a percentage of total PARP protein is shown in the absence (○) or presence (●) of IGF-1. Bars, SEM. Asterisks show PARP cleavage that is statistically significantly less in the presence of IGF-1 than in its absence.

proteins. MDA-MB-231 cells express relatively low levels of Akt, whereas MDA-MB-468, Hs 578T, and BT-549 cells express relatively higher levels of Akt. Similarly, the relatively high levels of phosphorylated MAPK detected after IGF-1 treatment, in MDA-MB-468, MDA-MB-435s, and even higher levels in HBL-100 cells, may be partly because MAPK expression is relatively high in these cell lines.

Our results suggest that higher concentrations of downstream effector proteins may, in part, compensate for low receptor concentrations to allow a triple-negative breast cancer cell with low IGF receptor concentrations to respond to IGF-1.

### *Concentration Dependence of the IGF-1 Effect on the Phosphorylation of Signaling Proteins, Cell Proliferation, and Cell Survival*

Although IGF-1 stimulates proliferation and provides robust protection from induction of cell death in cells that express high and low levels of IGF receptors, the receptor level could influence the sensitivity to IGF-1, and this could have implications for IGF-targeted therapy. We therefore investigated the hypothesis that differing levels of receptor expression and phosphorylation control the sensitivity of the biologic response to IGF-1.



**Figure 5.** Phosphorylation of IGF receptors, Akt and MAPK, in response to IGF-1 in triple-negative breast cancer cells. BT-20, MDA-MB-231, MDA-MB-468, MDA-MB-435s, Hs 578T, BT-549, HBL-100, and SK-BR-3 cells were seeded in routine culture medium, cultured for 2 days in phenol red-free medium supplemented with charcoal-treated serum, for 2 hours in serum-free medium, and then stimulated with 50 ng/ml IGF-1 for 15 minutes in the same medium, and protein lysates were prepared. Aliquots of 10  $\mu$ g of protein were electrophoresed on a polyacrylamide gel; transferred to nitrocellulose; incubated with phospho-IGF receptor, phospho-Akt, or phospho-ERK1/2 antibodies; and developed using enhanced chemiluminescence as described in the Materials and Methods. Because we detected exceptionally high amounts of phosphorylated Akt and MAPK in BT-20 cells and of phosphorylated MAPK in HBL-100 cells, lower exposures of the membranes to the x-ray films are shown for these proteins. The membranes were incubated secondly with a tubulin antibody to confirm equal loading and transfer. The protein samples were analyzed to measure the total level of the proteins and to confirm that the levels do not change during the experiment. Representative images of the results obtained are shown underneath the panels of the corresponding phosphorylated proteins.



The concentration dependence of IGF-stimulated receptor phosphorylation was measured in MDA-MB-231 cells that express high levels of the type I IGF and insulin receptors, in MDA-MB-468 cells that express significant levels of the type I IGF receptor only, and in Hs 578T cells that express low levels of both receptors (Figure 6). In all three cell lines, the amount of phosphorylated receptor detected was near-maximal at 25 ng/ml IGF-1 and half-maximal at around 6 ng/ml IGF-1. Attenuation of receptor phosphorylation at high ligand concentrations was not detected in any cell line. The important differences between the cell lines were that *de facto* the total amount of phosphorylated receptor present after stimulation with saturating concentrations and subsaturating concentrations varied, reflecting the level of expression of the receptor. Also, the fold increase in the amount of phosphorylated receptor detected at 0.5 ng/ml IGF-1 and 25 ng/ml IGF-1 differed. This fold difference was more than 1000-fold for MDA-MB-231 cells, more than 100-fold for MDA-MB-468 cells, and approximately 6-fold for Hs 578T cells.

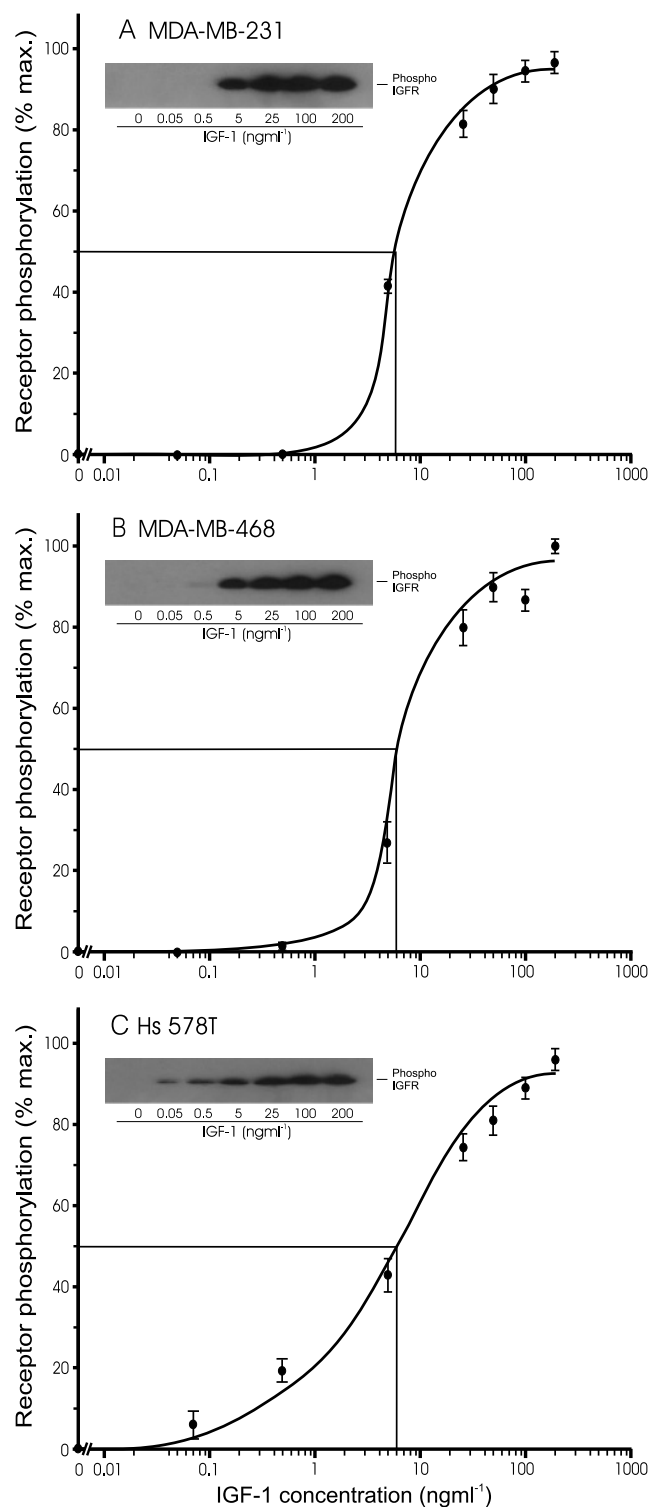
We next investigated the concentration dependence of phosphorylation of Akt and MAPK, proliferation and cell survival in MDA-MB-231, which express high levels of receptor, and Hs 578T cells, which express low levels. Both Akt and MAPK were phosphorylated at lower IGF-1 concentrations in MDA-MB-231 than in Hs 578T (data not shown). There was a clear proliferative response of both cell lines to low IGF-1 concentrations, but MDA-MB-231 cells were more sensitive than Hs 578T cells (Figure 7, A and B). IGF-1 increased MDA-MB-231 cell proliferation at 50 pg/ml, whereas an increase in Hs 578T cell number was only significant at concentrations of 2 ng/ml IGF-1 and higher. The magnitude of the proliferative effect increased to reach a maximum at 50 ng/ml IGF-1 for MDA-MB-231 cells and 100 ng/ml IGF-1 for Hs 578T cells. The proliferative effect of IGF-1 in MDA-MB-231 cells was attenuated somewhat at concentrations greater than 50 ng/ml.

For cell survival, there was a clear response of both cells lines to low IGF-1 concentrations (Figure 7, C and D). A protective effect of IGF-1 was first detected in MDA-MB-231 cells at 50 pg/ml IGF-1. The protective effect reached a maximum at around 10 ng/ml IGF-1 but was attenuated at higher concentrations. In Hs 578T cells, a protective effect of IGF-1 was detected at concentrations greater than 500 pg/ml. The magnitude of the IGF-1 survival effect increased with higher IGF-1 concentrations to reach a maximum around 200 ng/ml IGF-1.

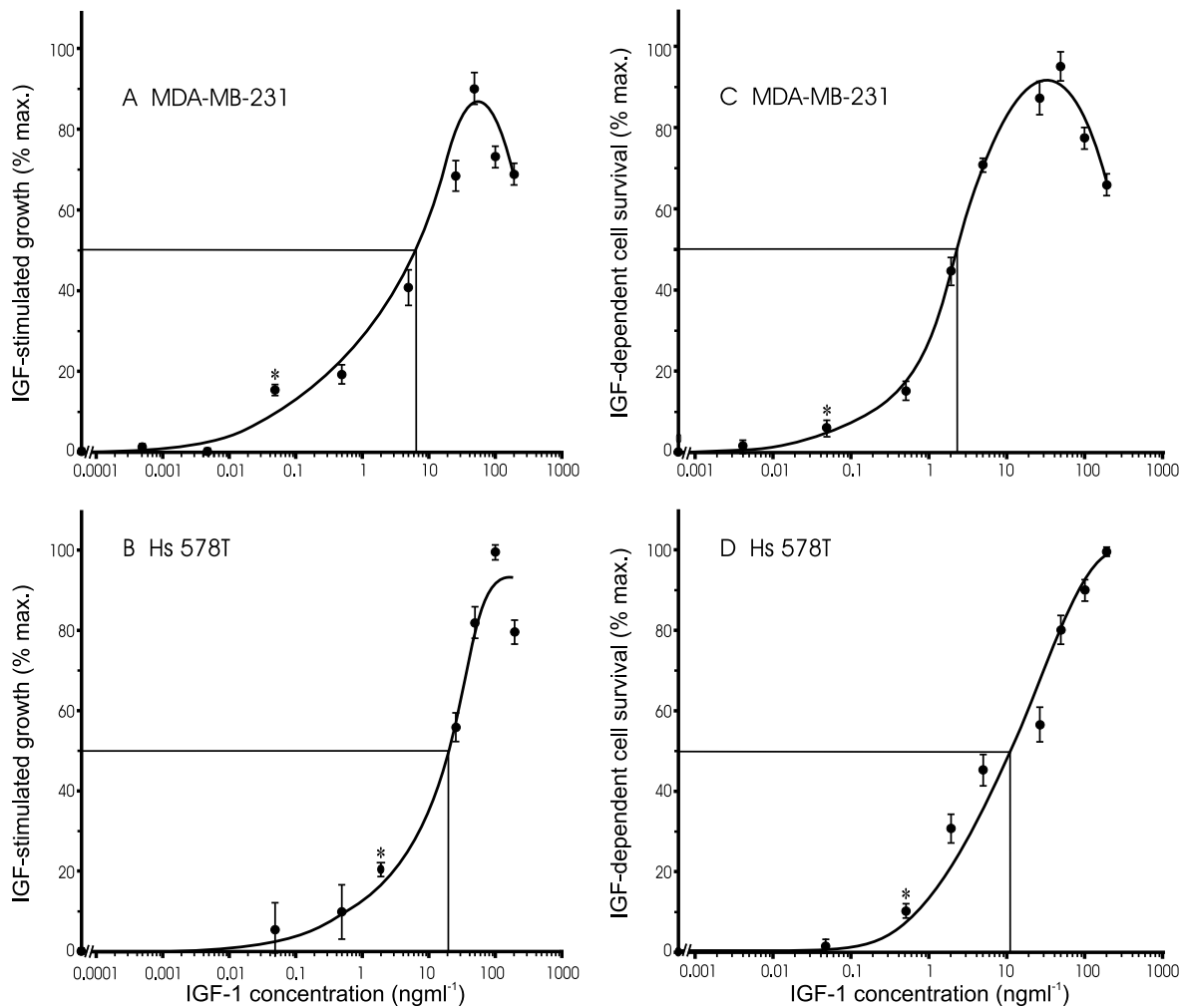
These results show that the growth and survival of triple-negative breast cancer cells with very different concentrations of type I IGF receptor is increased by low IGF-1 concentrations. The sensitivity to IGF-1-stimulated autophosphorylation of the type I IGF receptor cells is the same for cells that express high and low receptor concentrations, but cells that express higher levels of receptor are more sensitive for the activation of downstream signaling molecules and for the induction of biologic responses.

## Discussion

All breast cancer cell lines expressed type I IGF receptor irrespective of their estrogen receptor status, and there was no association between type I IGF receptor expression and estrogen responsiveness. This is unexpected because type I IGF receptor expression is increased by estrogen in estrogen-responsive breast cancer cells [23,35] and is associated with estrogen receptor expression in large series of breast tumors [43,44]. It is in agreement, however, with early studies that showed by ligand binding that similar amounts of IGF receptor are expressed on



**Figure 6.** Stimulation of phosphorylation of IGF receptors by different concentrations of IGF-1. MDA-MB-231 (A), MDA-MB-468 (B), and Hs 578T (C) cells were withdrawn for 2 days and then incubated in the presence of the indicated concentrations of IGF-1 for 15 minutes. Cells were lysed, and phosphorylation of IGF receptors was measured and quantified as described in the Materials and Methods. Representative images from the membranes after exposure to an x-ray film for 1 minute (MDA-MB-231), 10 minutes (MDA-MB-468), and 5 hours (Hs 578T) are shown as inserts. The amount of phosphorylated receptor was determined by densitometric scanning of the x-ray films followed by analysis with LabWorks 4.0 software. Bars, SEM.



**Figure 7.** Stimulation of cell proliferation and cell survival by different concentrations of IGF-1. MDA-MB-231 (A) and Hs 578T (B) cells were withdrawn from growth stimuli and cultured in the presence of the indicated concentrations of IGF-1. Cells were lysed, and the DNA content of the wells was measured as described in the Materials and Methods. MDA-MB-231 (C) and Hs 578T (D) cells were treated with staurosporine in the absence or presence of the indicated concentrations of IGF-1. Cells were lysed, and the percentage of PARP cleavage was determined as described in the Materials and Methods. Bars, SEM. Asterisks indicate the lowest concentration of IGF-1 at which a significant effect of IGF-1 was detected.

the cell surfaces of estrogen-unresponsive MDA-MB-231 cells and estrogen-responsive MCF-7 cells treated with estrogen [23]. Others studies have measured type I IGF receptor in triple-negative breast cancer cell lines notably in MDA-MB-231 cells [28,29,45] but have not compared type I IGF receptor expression in the different triple-negative breast cancer cell lines described in the present study.

IGF receptor phosphorylation was stimulated by IGF-1 in all triple-negative cell lines tested. Few studies have measured phosphorylated IGF receptor in breast tumors. The study of Law et al. [46] is of interest because it suggests that the level of phosphorylated receptor is indicative of poor prognosis, whereas previous studies suggested that total type I IGF receptor levels were of no prognostic value [47] or were indicative of good prognosis [43].

We demonstrate that the IGF signal transduction pathway is active in triple-negative breast cancer cells and that its activation increases cell proliferation and promotes cell survival in all the triple-negative breast cell lines tested. Further, decreased type I IGF receptor expression reduced cellular proliferation of triple-negative cells. Supportive of the clinical relevance of our findings, IGF inhibitors were effective in a

mouse model of basal cell mammary cancer [48], an IGF gene signature has been reported in triple-negative breast tumors [49], and Law et al. [46] found that phosphorylated IGF receptors were indicative of poor prognosis in all breast cancer types.

The effects of IGFs on the proliferation of estrogen-unresponsive, triple-negative breast cancer cells are controversial. Our early study reported that the growth of MDA-MB-231 cells is increased by IGF-1 [23], but subsequent studies have reported no effect in MDA-MB-231 [28], MDA-MB-468 [29,30], MDA-MB-435A [30], and Hs 578T cells [31]. The reasons for the discrepancies are not known, but the culture conditions used for the proliferation assays may be critical. It may be important that cells are withdrawn from the stimulatory effect of IGF-1 and high concentrations of insulin present in most routine cell culture medium. It may be important that cells are not cultured in a minimal, serum-free medium in which cells may be refractory to stimuli. Other explanations for the different results obtained in the present study and those reported by other workers are the different densities of the cells and the frequency of medium change, which may affect the impact of molecules released by the cells themselves [31]. It

may also be relevant that most studies have tested the effects of IGFs over relatively short times, whereas experiments were more prolonged in the present study. After 3 days of IGF-1 treatment, the discernible increase in cell number was either small or not significant. Although the magnitude of the effect varied, cell growth was stimulated by IGF-1 in all five triple-negative breast cancer cell lines studied. Interestingly, whereas the greatest effect on proliferation occurred in a cell line that expresses high concentrations of IGF receptors, one of the highest fold increases in cell number stimulated by IGF-1 occurred in Hs 578T cells, which contain relatively low concentrations of IGF receptors and low IGF-1 stimulation of IGF receptor phosphorylation.

The protective effects of IGF-1 against induction of triple-negative breast cancer cell death reported in this study are dramatic. The effect of IGFs on cell survival of triple-negative breast cancer cells has not been reported. Similarly to cell proliferation, the ability of IGF-1 to inhibit induction of cell death was not affected markedly by the amount of expression or phosphorylation of the IGF receptors.

Our results suggest that, in addition to the concentrations of the IGF receptors, concentrations of downstream effector proteins may contribute to the ability of triple-negative breast cancer cells to respond to IGFs. The expression levels of adaptor proteins could contribute also to IGF responsiveness especially in cells such as Hs 578T that have relatively low concentrations of IGF receptors. We have shown recently that both Hs 578T and HBL-100 cells express relatively high levels of the major IGF receptor adaptor protein, insulin receptor substrate 1 (IRS-1) compared with MDA-MB-231 cells [35]. One explanation for the robust response of Hs 578T cells and HBL-100 cells to IGF-1 is that they express high levels of IRS-1 [35]. This possibility is supported by our observation that the amount of IRS-1 phosphorylated on Tyr 896 is very high in Hs 578T cells stimulated with IGF-1 and relatively high in HBL-100 cells stimulated with IGF-1 (data not shown), which indicates that strong induction of IRS-1 tyrosine phosphorylation in response to IGF-1 in Hs 578T and HBL-100 cells may contribute to the response of these cells to IGF-1. By contrast, serine phosphorylation of IRS-1 attenuates IGF receptor signal transduction [50], and it is possible that differential serine phosphorylation of IRS-1 may influence the responsiveness to IGF-1 of the different triple-negative breast cancer cell lines. It seems that the responsiveness of individual cells to IGFs results from the composite expression of different components of the IGF signal transduction pathway and is not determined solely on the basis of the level of expression of the IGF receptors.

The potential consequence of IGF receptor concentration in triple-negative breast cancer cells on their biologic responsiveness to IGFs was investigated further by measuring phosphorylation of IGF receptors, Akt and MAPK, growth stimulation, and protection from induction of cell death effected by different concentrations of IGF-1 in cells that express high and low receptor concentrations. Our results show that, whereas the concentration dependence of the induction of IGF receptor phosphorylation is similar regardless of the number of receptors present in the cells, the concentration of IGF-1 required to elicit biologic responses was higher in cells that express lower receptor levels. These observations may have therapeutic implications because they suggest that a higher dose of an IGF-1 antagonist would be required to inhibit tumor cells with high receptor levels. A threshold level of phosphorylated receptor may be required to elicit a biologic response, and this threshold is achieved at lower IGF-1 concentrations in cells that contain high receptor levels than in cells that contain low receptor levels. Importantly, however, even cells that express small amounts of

IGF receptors are protected from cell death, and their proliferation increased in the presence of relatively low IGF-1 concentrations.

There is a dearth of treatment options for triple-negative breast cancers because their phenotype renders them unresponsive to hormonal or HER2-targeted therapies [1,2,48]. To date, the emphasis has been on cytotoxic agents. They respond better than other types of breast cancer to neoadjuvant chemotherapy, but patients with residual disease tend to relapse and die earlier. Other therapeutic targets are being evaluated in triple-negative breast cancer. High levels of EGFR are expressed in triple-negative tumors. Current data suggest, however, that most triple-negative cancers respond transiently, if at all, to EGFR inhibition. Trials of vascular endothelial growth factor, Src, MEK, PARP, and histone deacetylase inhibitors have been initiated. These studies reflect current endeavor to provide a solution to the large clinical challenges presented by triple-negative breast cancers.

Although the IGF signal transduction pathway has attracted considerable interest as a therapeutic target for the treatment of cancer [17,52], current trials of IGF signal transduction pathway inhibitors in breast cancer are in combination with existing agents on hormone responsive or HER2-positive cancers. IGF inhibitors are not being tested on triple-negative breast cancers because there have been insufficient data from preclinical models to indicate that triple-negative breast cancer cells are IGF-responsive.

The present study demonstrates that not only are the receptors and downstream signal transduction proteins present and phosphorylated in response to IGF-1 but that triple-negative breast cancer cell proliferation and survival, two key hallmarks of a cancer cell, are stimulated by IGF-1. Some of the cell line models in this study were established from young (HBL-100, MDA-MB-435s, MDA-MB-468, MDA-MB-231) and black (MDA-MB-468) women who are more likely to develop triple-negative breast cancer. In addition, the cell lines were established from primary tumors and metastatic cells. Our results suggest, therefore, that therapies targeted against the IGF signal transduction pathway could provide part of the arsenal to fight triple-negative breast cancer and could be used for the treatment of residual primary and systemic disease.

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