Tumorigenicity of MCF-7 human breast cancer cells lacking the p38 α mitogen-activated protein kinase

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Abstract

We have generated cell lines with significantly reduced expression of the p38 mitogen-activated protein kinase (p38 MAPK), Min-p38 MAPK cells, and used these cells to investigate p38 MAPK's role in tumorigenesis of breast cancer cells. MCF-7 cells were stably transfected with a plasmid producing small interfering RNA that inhibited the expression of p38 MAPK. Control cells were stably transfected with the same plasmid producing non-interfering RNA. The reduction in the p38 MAPK activity caused a significant increase in the expressions of estrogen receptor- α (ER α) and the progesterone receptor, but eliminated the expression of ER β . Min-p38 MAPK cells showed an enhanced overall growth response to 17 β -estradiol (E₂), whereas GH plus epidermal growth factor were largely ineffective growth stimulators in these cells compared to

controls. Although the long-term net growth rate of the Min-p38 MAPK cells was increased in response to E₂, their proliferation rate was lower compared to controls in short-term cultures. However, the Min-p38 MAPK cells did show a significant decreased rate of apoptosis after E₂ treatment and a reduction in the basal phosphorylation of p53 tumor suppressor protein compared to controls. When the Min-p38 MAPK cells were xenografted into E₂-treated athymic nude mice, their tumorigenicity was enhanced compared to control cells. Increased tumorigenicity of Min-p38 MAPK cells was caused mainly by a decrease in the apoptosis rate indicating that the lack of the p38 MAPK caused an imbalance to increase the ER α :ER β ratio and a reduction in the activity of the p53 tumor suppressor protein.

Journal of Endocrinology (2011) **208**, 11–19

Introduction

Estrogen and insulin-like growth factor 1 (IGF-I) are both central to breast development (Kleinberg & Ruan 2008), and evidence indicates that both these hormones affect carcinogenesis of the breast (Fagan & Yee 2008). Although both of these hormones are most commonly associated with cell growth stimulation and anti-apoptosis, they will also induce apoptosis under certain conditions. For example, a domain in the C-terminus of the IGF-I receptor (IGF-IR) has been found to have an apoptotic activity (Liu *et al.* 1998), and apoptotic activity of estrogen under certain physiological circumstances is now well documented (reviewed in Song & Santen (2003)). However, although we know that estrogen and IGF-I interact closely to regulate mammary gland development, many aspects of these interactions are not well understood. We recently showed (Mendoza *et al.* 2011) that lowering the expression of IGF-IR caused a decrease in the expression of the estrogen receptor- α (ER α), increased expression of ER β , and significantly enhanced the apoptosis rate of breast cancer cells. Furthermore, we demonstrated that when the cells with low expression of the IGF-IR were treated with 17 β -estradiol

(E₂), a rapid (within 15 min) increase in activation of the p38 mitogen-activated protein kinase (p38 MAPK) was seen when compared to controls with intact IGF-IR expression. These results indicate that the reduced level of the IGF-IR caused a shift towards lowering the ER α :ER β ratio. The hypothesis that the ratio of the two ERs might be important for function is not new. Hall & McDonnell (1999) proposed some 10 years ago that relative levels of the two ERs determined the transcriptional activity of the ERs with ER β playing a modulatory role on ER α activity under conditions of limited concentration of the ligand. More recently, Chang *et al.* (2006) demonstrated the modulatory role of ER β on ER α transcriptional activity in breast cancer cells. Less is known about the interactions of the two ERs at the plasma membrane to induce non-genomic, rapid action. However, both ER α and ER β are capable of exerting non-genomic activity (Razandi *et al.* 1999, 2004, Pedram *et al.* 2006), and they appear to be acting through different signaling pathways and elicit opposite affects, with ER α activating the extracellular signal-regulated kinase pathway possibly in association with IGF-IR–matrix metalloproteinases–heparin-binding epidermal growth factor (EGF) to stimulate growth (Song *et al.* 2007)

and ER β activating the p38 MAPK to increase apoptosis (Acconcia *et al.* 2005). As mentioned above, we recently generated breast cancer cell lines with reduced expression of IGF-IR (Mendoza *et al.* 2011). We found that these cells showed a decreased growth potential when stimulated with hormones, an increase in the expression of ER β , while ER α levels were reduced. Concomitant with these changes was an increase in the rate of apoptosis and elevated phosphorylation of p38 MAPK in response to estrogen treatment. We speculated that the reduced ER α :ER β ratio caused the increase in the p38 MAPK activation upon E₂ treatment and an increase in the rate of apoptosis, probably through the p53 tumor suppressor protein. To investigate this further, we have now generated cell lines with impaired expression of the p38 MAPK, and here we investigated the effects of this impairment on tumorigenicity of these breast cancer cells.

Materials and Methods

Cells

The MCF-7 human breast cancer cells were obtained from ATCC (Manassas, VA, USA) and maintained at 37 °C in 90–95% humidity with 5% CO₂ in phenol red-free DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 50 µg/ml gentamicin, basic growth medium. For experimentation, the cells were plated in DMEM/F12 without phenol red and containing 10% FBS. After overnight culture, the medium was changed to serum-free, phenol red-free, DMEM/F12 containing trace elements and cultured for an additional 24 h. The cells were then exposed to different treatments and for varying lengths of time, as indicated in the figure legends.

Stable transfection of MCF-7 cells

To block or significantly reduce the p38 α MAPK expression, custom-made expression vectors capable of generating small double-stranded interfering RNA (siRNA) corresponding to a 21 nucleotide (nt) sequence of the human p38 α MAPK cDNA sequence were obtained from InvivoGen (San Diego, CA, USA). The control vector carried sequence generating non-interfering, 21 nt RNA (InvivoGen). MCF-7 cells were plated onto 6-well plates in DMEM/F12 medium containing 10% FBS and 50 µg/ml gentamicin at ~60% confluency. They were incubated for 24 h, and then the medium was changed to DMEM/F12 without serum and antibiotics. LipofectAMINE PLUS (Invitrogen) was used for the transfection according to the manufacturer's instructions. Approximately 24 h after the transfection, fresh medium containing 10% FBS was added, and the cells were incubated for an additional 48 h. At that time, the cells were exposed to medium containing the selectable marker (5 µg/ml blasticidin, Fisher Scientific, Pittsburgh, PA, USA). Viable cell colonies were localized and isolated with clone rings. These were

cultured in medium containing 10% FBS and blasticidin. The same cloning procedure was used for the control cells that had been transfected with the inactive vector.

Cell number studies

The changes in the number of the cells lacking functional p38 MAPK (Min-p38 MAPK) were studied in a defined medium. The cells were plated in 24-well culture plates at the density of 50 000 cells/well in DMEM/F12 medium containing 10% FBS and antibiotics and cultured overnight. The cells were then placed in serum-free, phenol red-free medium and cultured for an additional 24 h. At that time, media containing trace elements (MP Biomedicals, Solon, OH, USA) and different treatments were applied. Control cells were cultured in parallel with the Min-p38 MAPK cells using the same treatments and for the same length of time. Each treatment was continued for 6 days, and the media replenished every other day. The net changes in cell numbers were assessed by measuring the total DNA of the cells using the diaminobenzoic acid method (Hinegardner 1971).

5-Bromo-2'-deoxyuridine incorporation

ELISA assay (Roche Applied Science) based on 5-bromo-2'-deoxyuridine (BrdU) uptake was used to measure the short-term (18 h) proliferation rate of the cultured cells. For the assay, the cells were plated onto 96-well plates in 10% FBS and incubated for 24 h. Medium was then changed to serum free, and incubation continued for an additional 24 h when the cells were exposed to the different treatments. The treatments were continued overnight, and the following morning the media were removed and fresh treatment media containing 10 µM BrdU were applied to the cells and incubation continued for an additional 90 min. The cultures were then terminated, and BrdU incorporation was measured according to the manufacturer's instructions.

Apoptosis assay

The rate of apoptosis was measured using an ELISA (M30-Apoptosense, Peviva, Bromma, Sweden). This assay utilizes a specific antibody that was generated against a neo-epitope on cytokeratin 18 that is exposed after caspase cleavage (Hagg *et al.* 2002). For the assay, cells were plated onto 96-well plates in DMEM/F12 medium containing 10% FBS and incubated overnight. The cells were then serum-starved for an additional 24 h and then treated overnight with different hormones. To assess the total number of viable cells at the time each treatment began, replicate wells were treated with 60 µM roscovitine, a concentration that has been shown to cause complete cell apoptosis (Schutte *et al.* 2004). Experiments were terminated by lysing the cells using 10% NP-40, final concentration 0.5%, and the cell lysate was then diluted and assayed according to the manufacturer's directions.

Western blotting

The expression of the p38 MAPK, ER α , ER β , the progesterone receptor (PR), the total p53 protein level, and cyclin D1 were assessed using western blotting. The cells were plated onto 60 mm diameter culture dishes in the DMEM/F12 medium containing 10% FBS at the density of 4.0×10^6 cells/dish and incubated overnight. The medium was then changed to serum free, and the incubation was continued for an additional 24 h when cells were treated with different treatments and cultured for an additional 24 h. The cells were then scraped and lysed, total protein was extracted, and protein concentrations were measured using the BCA assay (Pierce, Rockford, IL, USA). After electrophoresis, the protein was transferred to polyvinylidene fluoride (PVDF) membrane for western analysis using chemiluminescent detection. The ER α -specific antibody was from Thermo Fisher Scientific (Fremont, CA, USA), and antibodies to ER β , PR, p38 MAPK, p53, cyclin D1, and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescent detections and densitometric quantifications were carried out using Kodak Image Station 2000R (Eastman Kodak Company), and the results for each protein are expressed as a ratio of β -actin expression. For each quantitative analysis, the results from two cell lines in each group (controls, Min-p38 MAPK) were pooled. The total number of observations is indicated in the figure legends for all western blots.

ELISA assays for measuring phosphorylation of p53

Phosphorylation level of the tumor suppressor protein p53 was measured using an ELISA (R&D Systems, Inc., Minneapolis, MN, USA). This assay is specific for detecting the phosphorylation of serine 46 on the p53 tumor suppressor protein. The cells were plated onto 60 mm diameter culture dishes in DMEM/F12 medium containing 10% FBS and cultured for 24 h. The cells were then serum starved for an additional 24 h, and then the different treatments were applied followed by 10 min incubation at 37 °C when the reaction was stopped by adding 5 ml ice-cold PBS. After washing, the cells were harvested and lysed, and the lysate was assayed according to the manufacturer's instructions. Total protein concentrations (BCA, Pierce) of the cell lysates were used for normalizing the assay results. Results for the phosphorylation of p53 in Min-p38 MAPK cells 1 and 2 were combined for statistical analysis and compared to control cells.

Xenografting of the Min-p38 MAPK and control cells

The cells were plated onto 75 cm² culture plates in DMEM/F12 medium containing 10% FBS and cultured to confluency. The cells were then harvested by scraping, counted, and suspended in Matrigel (BD Biosciences, Bedford, MA, USA) at a density of 1×10^7 cells per 150 μ l. The athymic nude mice were anesthetized and then inoculated with 1×10^7 cells in 150 μ l Matrigel. The animals

received two s.c. inoculations, one in each flank, one with Min-p38 MAPK cells and the other with scrambled siRNA vector control cells. The animals were then given s.c. silastic capsule implants (Thordarson *et al.* 2004) containing 30 μ g E₂. The mice were inspected weekly for detection of tumors, and all new tumors that were detected were measured using calipers, beginning 1 month after inoculation. The animals were terminated 2 months after inoculation; tumors were harvested, weighed, and fixed in 10% formalin for histology and immunostaining. All care and use of the animals in this study was approved by the Animal Care Committee at Texas Tech University Health Sciences Center.

Histology

The formalin-fixed tissues were paraffin embedded and sectioned into 5 μ m sections. The sections were deparaffinized, rehydrated, and then subjected to antigen retrieval using Trilogy (Cell Marque, Rocklin, CA, USA) and pressure cooker procedure. After blocking with 5% BSA, and avidin and biotin blocking agents, the tissue sections were exposed to

Figure 1 Western blot analysis showing expression of the p38 mitogen-activated protein kinase (p38 MAPK) and β -actin (internal control) in cloned MCF-7 cells stably transfected with small interfering RNA (siRNA) to the p38 MAPK (Min-p38 MAPK1, 2), and control clones (Control1, 2) transfected with inert small RNA of the same size as the p38 MAPK-siRNA. Cells were plated onto 60 mm culture dishes in DMEM/F12 medium containing 10% FBS and cultured for 24 h, followed by serum starvation for an additional 24 h, when cells were harvested and lysed, and total protein was extracted and measured using BCA assay. Samples were electrophoresed using 60 μ g total protein per lane and western blotted (A). The protein levels of p38 MAPK were quantified using densitometric analysis, and the results are expressed as p38 MAPK/ β -actin ratio. Each bar represents the mean $\pm$ S.E.M. of four replicates for both groups. Asterisk indicates significant difference between controls and Min-p38 MAPK cells, $P < 0.05$ (B).

Figure 2 The effects of 100 nM 17 β -estradiol (E₂), and a combination of 500 ng/ml human GH and 10 ng/ml epidermal growth factor (EGF) on growth of MCF-7 cells stably transfected with siRNA to the p38 MAPK (Min-p38 MAPK1, 2) and controls with intact expression of p38 MAPK. The cells were plated onto 24-well plates in DMEM/F12 medium containing 10% FBS and incubated for 24 h, followed by 24 h serum starvation and then treated for 6 days. Medium was replenished every other day. At the end of the culture period, total DNA was measured to assess overall cell growth. Each bar represents the mean $\pm$ S.E.M. for four replicate wells. *Significantly different, $P < 0.05$.

primary antibody (p38 α MAPK, Santa Cruz Biotechnology) for 1 h. Immunostaining was detected using Mouse/Rabbit PolyScan HRP/DAB Detection System (Cell Marque).

Statistical analysis

Data are presented as means $\pm$ S.E.M. of three to eight observations. Significant levels between groups were determined using ANOVA and Student–Newman–Keuls *post-hoc* test. $P < 0.05$ was considered statistically significant.

Results

Transfection

Western blot analysis showed that stable transfection with plasmid carrying siRNA with homology to the p38 MAPK resulted in two clones with undetectable or very low expression of the p38 MAPK compared to controls transfected with inert plasmid (Fig. 1A and B). Both these clones (Min-p38 MAPK1 and Min-p38 MAPK2) were used in all subsequent experimentations. Several other clones that survived the blasticidin selection did not show a significant reduction in the p38 MAPK expression and, therefore, were not used for further studies. Two controls that were stably transfected with plasmid carrying non-interfering RNA showed characteristics identical to those of intact MCF-7 cells in terms of a net increase in cell number, proliferation, and apoptosis rates of untreated and hormonally treated cells. Therefore, one representative control is presented in subsequent studies except when otherwise indicated.

Growth characteristics of the Min-p38 MAPK cells

Figure 2 shows that the absence of p38 MAPK expression affected overall growth rate of the Min-p38 MAPK cells. In particular, when they were treated with E₂ for 6 days, they showed a significantly higher net increase in cell number compared to controls, whereas their increase in cell number was decreased compared to control cells when they were treated with human GH plus EGF. In fact, although a slight increase in cell number was seen after GH plus EGF treatment, this increase was not significantly different from that seen in untreated Min-p38 MAPK cells. Although the net increase in cell number of the Min-p38 MAPK cells was higher than that of controls after E₂ administration, this was not reflected in an increase in cell proliferation after a short-term culture in that when the Min-p38 MAPK cells were treated with E₂ for 18 h, their BrdU uptake was actually lower than that of controls (Fig. 3). These results indicate either that more than 18 h were needed for the Min-p38 MAPK cells to significantly enhance their proliferation rate over controls, or that their apoptosis rate was reduced compared to controls. Indeed, when we measured the apoptotic rate of the Min-p38 MAPK cells, they did show a significant reduction in programmed cell death compared to controls (Fig. 4). Therefore, it is likely that the main cause for the net increased cell number of the Min-p38 MAPK cells after E₂ stimulation was a decrease in cell death but not increased proliferation. However, apoptosis rate was not affected by the E₂ treatment in either Min-p38 MAPK cells or controls, but it was probably the reduction in the basal apoptosis rate seen in the

Figure 3 Proliferation rate of the MCF-7 cells expressing a low level of p38 mitogen-activated protein kinase (Min-p38 MAPK1, 2) and controls. The cells were plated onto 96-well plates in DMEM/F12 medium with 10% FBS and incubated overnight. The following day, the medium was changed to serum free, and the cells were incubated for an additional 24 h. The cells were then treated with 100 nM 17 β -estradiol (E₂) or vehicle (untreated) and incubated overnight. Fresh media containing 10 μ M 5-bromo-2'-deoxyuridine (BrdU), treatment and control were then added, and the incubation continued for an additional 90 min when cultures were terminated and BrdU uptake was determined. Each bar represents mean $\pm$ S.E.M. for eight replicate wells. *Significantly different, $P < 0.05$.

Figure 4 The rate of apoptosis was measured using an ELISA specific for a neo-epitope on cytokeratin 18 that is exposed after caspase cleavage. Min-p38 MAPK1, 2 and controls were plated onto 96-well plates in DMEM/F12 medium containing 10% FBS and incubated overnight. The cells were then serum-starved for an additional 24 h and then treated overnight with 100 nM 17 β -estradiol (E₂), 60 μ M roscovitine, or vehicle (untreated). Experiments were terminated by lysing the cells with 10% NP-40, final concentration was 0.5%, and the cell lysate was then diluted and assayed according to the manufacturer's directions. Each bar represents mean $\pm$ S.E.M. for four to five replicate wells. *Significantly different, $P < 0.05$.

Min-p38 MAPK cells compared to controls that allowed a faster accumulative increase in the total cell number over time for the Min-p38 MAPK cells.

Expression of ER α , ER β , PR, and cyclin D1

Western blot analyses were used to investigate whether the increased response of the Min-p38 MAPK cells to E₂ was associated with changes in the expression of the ERs. These analyses revealed that ER α expression in the Min-p38 MAPK cells was significantly enhanced when compared with controls, but the opposite was found for ER β , where the expression was barely detectable in Min-p38 MAPK cells (Fig. 5A and B). Concomitant with the increase in ER α expression was an increase in the A-form of the PR, but the B-form of the PR was found to be low in both controls and the Min-p38 MAPK cells (not shown). E₂ treatment enhanced slightly the PR-A expression in both Min-p38 MAPK cells and controls (Fig. 6A and B). However, the increase was not statistically significant in either controls or Min-p38 MAPK cells. Surprisingly, the expression of cyclin D1, another E₂-regulated gene, did not differ between the Min-p38 MAPK cells and controls (not shown).

Activity of p53 tumor suppressor protein

The p38 MAPK is an important regulator of the tumor suppressor protein p53 and is known to directly phosphorylate both serine 33 and serine 46 on the p53 protein (Bulavin *et al.* 1999, Takekawa *et al.* 2000). To investigate whether eliminating the expression of p38 MAPK would affect the activity of p53, the phosphorylation level of serine 46 was

measured. As shown in Fig. 7A, the basal phosphorylation state of p53 was significantly reduced in both the Min-p38 MAPK cell lines compared to control. To determine whether the reduction in the phosphorylation levels of p53 in the Min-p38 MAPK cells was caused by diminished total expression of p53 protein in these cells, a western blot analysis was carried out. As shown in Fig. 7B, no significant difference was seen in the total p53 protein expression between controls and the Min-p38 MAPK cells.

Tumorigenicity

Immunodeficient mice were xenografted with both the Min-p38 MAPK cells and controls. Five animals were inoculated; one flank with the Min-p38 MAPK cells and the other with controls. Tumors developed in all cases at the inoculation sites for both the Min-p38 MAPK cells and controls (Fig. 8A). However, growth rate of the xenografted Min-p38 MAPK cells was approximately fourfold higher than that of the control cells (Fig. 8B and C). As shown in Fig. 8D, the expression of the p38 MAPK remained low in the xenografted Min-p38 MAPK cell compared to controls demonstrating that the Min-p38 MAPK cell maintained the expression of the siRNA to p38 MAPK after transplantation.

Discussion

Recently, we found that lowering the expression of the IGF-IR caused a reduction in ER α expression but increased the levels of ER β and a concomitant decrease in growth of ER-positive breast cancer cells (Mendoza *et al.* 2011).

Figure 5 Western blot analysis showing basal expressions of the estrogen receptor α (ER α) and ER β in cloned MCF-7 cells stably transfected with small interfering RNA (siRNA) to p38 MAPK (Min-p38 MAPK) and controls. β -Actin was used as an internal standard. Cells were plated onto 60 mm culture dishes in DMEM/F12 medium containing 10% FBS and cultured for 24 h, followed by serum starvation for an additional 24 h, when cells were harvested and lysed, and total protein was extracted and measured using BCA assay, followed by SDS-PAGE using 60 μ g total protein per lane and western blotted (A). The results from the western blotting were quantified using densitometry and are presented as means $\pm$ S.E.M. of six (ER α for both groups) and four (ER β for both groups) replicates. Asterisk indicates significant difference between controls and Min-p38 MAPK cells, $P < 0.05$ (B).

Figure 6 Western blot analysis showing expressions of the progesterone receptor A (PR-A) in cloned MCF-7 cells stably transfected with small interfering RNA (siRNA) to p38 MAPK (Min-p38 MAPK) and controls. β -Actin was used as an internal standard. Cells were plated onto 60 mm culture dishes in DMEM/F12 medium containing 10% FBS and cultured for 24 h. The cells were then serum-starved overnight and then treated with 100 nM 17 β -estradiol (E₂) or vehicle (untreated) and incubated for an additional 24 h, when cells were harvested and lysed, and total protein extracted and measured using BCA assay. Samples were then subjected to SDS-PAGE using 60 μ g total protein per lane and western blotted (A). The results from the western blotting were quantified using densitometry and are shown as means $\pm$ S.E.M. of four replicates for both groups. Asterisk indicates significant difference between controls and Min-p38 MAPK cells, $P < 0.05$ (B).

An increase in the level of ER β has frequently been associated with an increased rate of apoptosis in different tissues (reviewed in Zhao *et al.* (2008)). We reasoned that this shift in the ER α :ER β ratio might be increasing the apoptosis rate of the cells and thereby decreasing their overall growth rate. Indeed, when we investigated this possibility, we found that programmed cell death was significantly increased in the cells with low IGF-IR expression and we also obtained evidence that this increased cell death was mediated through activation of the p38 MAPK (Mendoza *et al.* 2011). Here, we continue to study the involvement of p38 MAPK in cell death and survival. We generated cloned cell lines with significantly reduced expression of the p38 MAPK. In general, these cells displayed a phenotype completely opposite to that found in cells expressing a low level of IGF-IR in that they showed a higher ER α :ER β ratio, increased growth rate in response to E₂, decreased rate of apoptosis, and an increase in tumorigenicity when xenografted into immunodeficient mice. The role p38 MAPK plays in regulating cell growth is complex. It can be mitogenic in certain cell types and under certain physiological conditions, but it has also been known that

activation of p38 MAPK can suppress cell growth and increase apoptosis, such as in different tumors (Ono & Han 2000, Bulavin & Fornace 2004, Thornton & Rincon 2009). How, under normal circumstances, the activity of the p38 MAPK is regulated in the breast epithelia is not fully understood. We found, as mentioned above, that inhibiting the expression of IGF-IR causes a reduction in the expression of ER α and an increase in the levels of ER β , and treatment of these cells with E₂ causes a rapid phosphorylation of the p38 MAPK (Mendoza *et al.* 2011). Here, we show that eliminating the expression of ER β to barely detectable levels, while ER α expression was significantly enhanced compared to control cells. Both these results suggest that the ERs are essential

Figure 7 The basal activity (phosphorylation) of the p53 tumor suppressor protein was measured using ELISA. The Min-p38 MAPK1, 2 and control cells were plated onto 60 mm culture plates in 10% FBS and incubated overnight, followed by 24 h serum starvation. Medium was then removed, and fresh serum-free medium was applied and incubation continued for an additional 10 min, when the cells were washed and harvested by scraping in ice-cold PBS. After centrifugation for 5 min, the cells were lysed and the assays were carried out. Total protein concentrations of the cell lysates were used for normalizing the assay results. Each bar represents the mean $\pm$ S.E.M. of three replicate plates for each cell line. * Significant difference between Min-p38 MAPK1, 2 and controls, $P < 0.05$. The results for the two Min-p38 MAPK cell lines were combined for the statistical analysis and are presented combined in the graph (A). The total protein expression levels of p53 were determined using western blot analysis and densitometric quantification. The results are expressed as p53/ β -actin ratio. Each bar represents the mean $\pm$ S.E.M. of four replicates for both groups (B).

Figure 8 The tumorigenicity of MCF-7 cells lacking p38 MAPK (Min-p38 MAPK) was studied by s.c. injecting Min-p38 MAPK cells into immunodeficient mice fitted with silastic capsule containing 30 μ g 17 β -estradiol (E_2). Each animal was injected with 1×10^7 Min-p38 MAPK cells in BD Matrigel (150 μ l) and control cells in the same medium. The animals received Min-p38 MAPK cells in one flank and control cells with scrambled siRNA vector in the other flank, i.e. each animal was inoculated with both Min-p38 MAPK cells and control cells with scrambled siRNA vector. Tumor size was measured weekly for 4 weeks beginning 1 month after inoculation. Two months after inoculation, animals were killed, and tumors were harvested and weighed. Photograph showing a mouse bearing Min-p38 xenograft and control cells with scrambled siRNA vector (A). Photograph showing xenografts of Min-p38 MAPK and control cells with scrambled siRNA vector after 8 weeks of growth. Each bar represents the mean $\pm$ S.E.M. of tumors from five animals. Asterisk indicates significant difference between control cells with scrambled siRNA vector and Min-p38 MAPK cells, $P < 0.05$ (C). Microphotograph showing immunohistochemical detection of p38 MAPK in tumor xenografts obtained from immunodeficient mice. 1) Positive immunostaining for p38 MAPK in control tumor with scrambled siRNA vector. 2) Section from the same control tumor with scrambled siRNA vector where the primary antibody was omitted (negative control). 3) Immunostaining for p38 MAPK of Min-p38 MAPK tumor. 4) Negative control (primary antibody omitted) for Min-p38 MAPK tumor. Note the weak immunostaining for p38 MAPK in the Min-p38 MAPK xenograft compared to the control xenograft tumors with scrambled siRNA vector (D). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0237>.

regulators of the p38 MAPK, and it appears that ER β is the active player in that regulation. We know that ER β will activate the p38 MAPK in the absence of ER α (Caiazza *et al.* 2007). In addition, evidence supports a dominant role of ER β in the interactions of the two ER isoforms (Pettersson *et al.* 2000, Liu *et al.* 2002). However, it has also been shown that ER α is capable of activating the p38 MAPK in complete absence of ER β (Lee & Bai 2002, Acconcia *et al.* 2005), and p38 MAPK phosphorylates ER α at threonine-311 (Thr³¹¹), demonstrating reciprocal interactions between p38 MAPK and ER α (Lee & Bai 2002). Therefore, our understanding of how the ERs regulate p38 activity is still incomplete. Nonetheless, increasing evidence now supports a significant role for ER β in enhancing cell apoptosis. For example, transfecting T47D ER-positive breast cancer cells with the ER β inhibits cell proliferation after E_2 administration (Strom *et al.* 2004), and ER β has been found to suppress the expressions of cyclin D1 and other growth regulatory genes in T47D breast cancer cells and HeLa cells (Liu *et al.* 2002, Strom *et al.* 2004). Also, transfecting MCF-7 breast cancer cells with ER β increases the efficacy of anti-estrogenic compounds in culture (Hodges-Gallagher *et al.* 2008). In addition, pre-invasive, proliferating breast cancers show a reduction in the

expression of ER β indicating a loss of growth inhibition (Roger *et al.* 2001), and reduced levels of ER β are associated with the anti-estrogen resistance in breast cancers (Hopp *et al.* 2004). We mentioned above that both ER α and ER β are capable of activating p38 MAPK. However, the effects of this p38 MAPK activation appear to be opposite. When Lee & Bai (2002) demonstrated that ER α causes phosphorylation of p38 MAPK and, in turn, p38 MAPK-phosphorylated Thr³¹¹ on the ER α , they associated this activity with an increase in cell growth, whereas ER β activation of the p38 MAPK is associated with an increase in the rate of apoptosis (Zhao *et al.* 2008), and, generally, most studies have shown the opposite effects of the two isoforms of the ERs on gene expression and cell growth in most cell types (Liu *et al.* 2002, Lindberg *et al.* 2003, Acconcia *et al.* 2005, Chang *et al.* 2006). Our results agree with these previous findings in that largely eliminating the expression of p38 MAPK causes a significant increase in growth of these cells in culture when treated with E_2 , and xenografting the cells into immunodeficient mice increased their tumorigenicity. Previously, we had demonstrated that an increase in p38 MAPK activity through a reduction in IGF-IR/ER α expression reduced growth rate in response to E_2 (Mendoza *et al.* 2011). How the two ERs are

causing phosphorylation of the p38 MAPK with opposite effects on cell growth is not understood. However, it is worth noting that p38 MAPK activation can be achieved through different signaling pathways (Ge *et al.* 2002, 2003, Kang *et al.* 2006, Lu *et al.* 2006) and, therefore, the two ERs could be utilizing different signaling for the activation of p38 MAPK resulting in opposite biological effects. This is certainly possible, particularly when results from experiments with different cell types are compared.

How p38 MAPK is increasing the apoptotic rate of the cells is not known. We found that the phosphorylation of the tumor suppressor protein p53 was significantly decreased in cells with inactive p38 MAPK, and previously we had shown that an increase in p38 MAPK activity was associated with an increase in p53 phosphorylation. It has been shown that p53 is a substrate for p38 MAPK (Bulavin *et al.* 1999, Takekawa *et al.* 2000), and we suggest a causative relationship between p38 MAPK activity, p53 phosphorylation, and rate of apoptosis in breast cancer cells. Recently, Chen *et al.* (2009) showed that inhibiting p38 MAPK activity in MDA-MB-468 and MDA-MB-231, both breast cancer cells with mutated p53 gene, inhibited growth of these cells, whereas MCF-7 cells with intact p53 gene (Casey *et al.* 1991) were not affected. They suggested that the effects of p38 MAPK in regulating growth of breast cancer depended on whether the tumors expressed intact or mutated forms of p53, with p38 MAPK predominantly enhancing apoptosis in cells with functional p53, but causing growth stimulation in cells lacking p53 function. Our results are in line with this conclusion and implicate the ERs as major players in this regulation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was generously supported by grants from Laura W Bush Institute for Women's Health-Permian Basin and National Institutes of Health/National Cancer Institute R03CA128067 and R03CA128067-02S1 awarded to GT.

Acknowledgements

We thank Edward Jin for providing many of the antibodies for this study and for useful discussions.

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Received in final form 19 October 2010

Accepted 25 October 2010

Made available online as an Accepted Preprint
25 October 2010