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TITLE: Mullerian Inhibiting Substance (MIS) Augments IFN-γ Mediated Inhibition of Breast Cancer Cell Growth

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13. ABSTRACT (Maximum 200 Words)

Mullerian Inhibiting Substance (MIS), a member of the TGFß family regulates growth, differentiation, and apoptosis in many cell types. In the male embryo, MIS causes regression of the Mullerian duct. However, the presence of MIS type II receptor gene in the breast is suggestive of its postnatal role in breast. Although the antitumor effects of IFN-γ are well documented, toxic effects associated with IFN-γ have precluded its use in the treatment of cancer patients. The purpose of this study is to test the hypothesis that MIS and IFN-γ might be more effective in the inhibition of breast cancer cell growth both *in vitro* and *in vivo* than either agent alone. Presently, we have observed that MIS and IFN-γ function through distinct molecular pathways. MIS induced NFκB DNA binding activity in breast cancer cells where as IFN-γ functions through Stat pathway as is observed using IkBα-DN clones. Co-stimulation of IRF-1 by MIS and IFN-γ in breast cancer cells is mediated through activation of NFkB and STAT pathways, respectively and is independent of Smad1 phosphorylation. The simultaneous addition of MIS and IFN-γ resulted in synergistic induction of CEACAM1 expression in T47D cells. In concordance with this observation, treatment of MDA-MB-468 cells with either MIS or IFN-γ inhibited growth and the presence of both inhibited growth better over a period of eight days. However, the enhanced inhibition of breast cancer cell growth by MIS and IFN-γ could not be explained by combined changes in cell cycle progression as both reagents alone significantly decreased the fraction of cells in the S-phase of the cell cycle, an effect not enhanced when they were used in combination.

14. SUBJECT TERMS
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REPORT CONTENT

Introduction:

MIS is a member of the TGFβ superfamily, a class of molecules that regulate growth, differentiation and apoptosis in many cell systems. The importance of MIS in the regression of the Mullerian duct in male embryos is well defined. However, the presence of MIS type II receptor gene, in other tissues including the breast is suggestive of its postnatal role. MIS inhibits breast cancer cell growth by interfering with cell cycle progression and inducing apoptosis. Our preliminary results demonstrate that MIS induced the interferon regulatory factor-1 (IRF-1) mRNA in breast cancer cells *in vitro* and *in vivo*. IRF-1 is also robustly induced by both type I and type II interferons. Although, the antitumor effects of Interferon-γ (IFN-γ) are well documented, toxic effects associated with IFN-γ have prohibited its use in the treatment of cancer patients. Our previous results demonstrated that IFN-γ costimulated MIS mediated induction of IRF-1, a gene known for its growth inhibitory functions. The enhanced gene expression by integration of MIS and IFN-γ induced signaling pathways may augment breast cancer cell growth inhibition. The objective of this study is to test the hypothesis that MIS and IFN-γ might be more effective in the inhibition of breast cancer cell growth both *in vitro* and *in vivo* than either agent alone.

Body (Results & Significance)

Specific Aim I: Characterization of the molecular mechanism that integrates IFN-γ and MIS mediated signaling. (12 months)

Task 1: To identify the molecular mechanism by which MIS and IFN-γ induce IRF-1 expression in breast cancer cells.

MIS and IFN-γ function through distinct molecular pathways.

MIS and IFN-γ induce IRF-1 expression in breast cancer cells. In order to identify the molecular mechanisms, by which MIS and IFN-γ induce IRF-1 expression, gel shift assays were carried out using NFkB or STAT-inducing element (SIE) oligonucleotides containing the relevant DNA binding consensus sequences. MIS induced NFkB DNA binding activity. The supershift experiments performed using anti p50 and anti p65 antibodies demonstrated that the complex consists of p50 and p65 NFkB subunits in T47D cells (figure 1a). Binding to the SIE DNA sequence was not observed suggesting that MIS does not evoke STAT DNA binding in these cells. IFN-γ however induced SIE DNA binding activity but did not activate the DNA binding activity of NFkB. Antibody supershift experiments demonstrated that the STAT-DNA protein complex induced by IFN-γ contained the STAT-1 protein but not STAT-3 or STAT-5a (figure 1b).

MIS and IFN-γ costimulate IRF1 expression through NFkB and STAT pathway.

In order to determine whether activation of the NFkB signaling cascade by MIS was responsible for the induction of IRF-1 mRNA, we generated T47D cell clones which express the dominant negative inhibitor of IkB (IkBα-DN). In the rat IkBα-DN transgene used in these experiments, two serine residues at positions 32 and 36 are replaced by alanines. Hence the resulting IkBα-DN protein cannot be phosphorylated in response to activation signals. Thus it functions as a super repressor of NFkB activation (Brown et al,

1995). T47D cell clone expressing the IkBα-DN transgene was identified by the lack of NFkB activation following MIS treatment (figure 2a). Induction of IRF-1 by MIS was greatly reduced in the clone harboring IkBα-DN compared to cells transfected with the empty vector (figure 2b). Thus MIS-induced IRF-1 requires activation of NFkB DNA binding activity. Overexpression of IkBα-DN in T47D cells did not interfere with induction of IRF-1 by IFN-γ. The stimulation of IRF-1 mRNA by a combination of MIS and IFN-γ was equivalent to that induced by IFN-γ alone since IRF-1 induction by MIS was impaired in these cells (figure 2b). Thus it is likely that co-stimulation of IRF-1 by MIS and IFN-γ in breast cancer cells is mediated through activation of NFkB and STAT pathways, respectively.

Task 2: To determine if MIS mediated activation of IRF-1 occurs via Smad pathway.

Induction of IRF1 by MIS is independent of the Smad pathway

The MIS typeII receptor, upon binding to the MIS ligand, initiates a signaling cascade that is dependent on recruitment of type I receptors, ALK2 and ALK6. Heterodimerisation of the type I and type II receptors induces the kinase activity of the type I receptor (Clarke et al, 2001; Gouedard et al, 2001) that subsequently phosphorylates the Smad1 protein. To investigate the contribution of Smad1 phosphorylation to MIS- mediated induction of IRF-1, T47D cells were transfected with a FLAG-tagged dominant negative (Smad1DN) construct in which serines at residues 462, 463 and 465 were converted to alanines. The transgene was stably transfected into T47D cells. Two clones expressing the Smad1DN gene were identified by northern blot (figure 3a). Similar levels of IRF-1 induction by MIS in vector and Smad1DN transfected T47D cells (figure 3b) demonstrated that MIS mediated induction of IRF-1 does not require phosphorylation of Smad1.

Task 3: To test the effect of MIS and IFN-γ on the gene expression of growth regulatory genes.

MIS and IFN-γ synergistically induce the expression of CEACAM1

CEACAM1 (carcinoembryonic antigen-realated cell adhesion molecule) also known as biliary glycoprotein (BGP) is a Ca^{2+} dependent cellular adhesion molecule that is expressed in epithelial cells (Thompson et al, 1994; Cheug et al, 1993). An interferon-sensitive response element (ISRE) in the CEACAM1 promoter is specifically protected by IRF-1 in DNA footprints and is required for induction of a CEACAM1 promoter-driven reporter construct by IRF-1 (Chen et al, 1996). Both MIS and IFN-γ induced CEACAM1 expression in T47D cells (figure 4). Interestingly, simultaneous addition of MIS and IFN-γ resulted in synergistic induction of CEACAM1 expression (figure 4).

The effect of MIS and IFN-γ on the expression of lysyl oxidase, p21 and IFNβ is under investigation.

Specific Aim II: Test the effect of MIS, IFN-γ, or both on breast cancer cell growth using *in vitro* and *in vivo* model systems. (24 months)

Task 4: To characterize the mechanism by which MIS and IFN-γ inhibit breast cancer cell growth.

Effect of MIS and IFN-γ on breast cancer cell growth

Since the signaling events initiated by MIS and IFN-γ converge to increase the magnitude of gene expression, we next tested their effect on the growth of breast cancer cells over a period of 1-8 days. Treatment of MDA-MB-468 cells with either MIS or IFN-γ inhibited growth and the presence of both inhibited growth better (figure 5a; n=8).

Alterations in cell cycle distribution are not responsible for MIS and IFN-γ induced growth inhibition.

In order to identify the mechanism by which MIS and IFN-γ inhibit growth. MDA-MB-468 cells were treated with MIS, IFN-γ or MIS+IFN-γ for 72 hours and the fraction of cells in each phase of the cell cycle was estimated by fluorescence activated cell sorting (figure 5B). Compared to untreated cells, MIS or IFN-γ treatment consistently led to a statistically significant decrease in the number of cells in the S-phase of the cell cycle ($p<0.001$ by Student's t-test). Interestingly, in cultures treated with a combination of MIS and IFN-γ, the percentage of cells in the S-phase did not demonstrate a greater decrease compared to that seen with either agent alone and these cultures did not exhibit any other extensive alteration in cell cycle distribution compared to cells treated with either agent alone. Thus the enhanced inhibition of breast cancer cell growth by MIS and IFN-γ could not be explained by combined changes in cell cycle progression compared to treatment with either agent alone.

Presently, we are working on the effect of MIS and IFN-γ on the expression of lysyl oxidase, p21 and IFN-β. In addition, the apoptosis assays will be carried out to test the mechanism by which MIS and IFN-γ inhibit the breast cancer cell growth. The next important aspect of this project is to determine whether the synergistic interaction between MIS and IFN-γ to suppress breast cancer cell growth *in vitro* can be recapitulated *in vivo*. To this end we will be testing various mouse models, and the model with most robust breast cancer growth will be used to test the effect of MIS and IFN-γ on the tumor regression.

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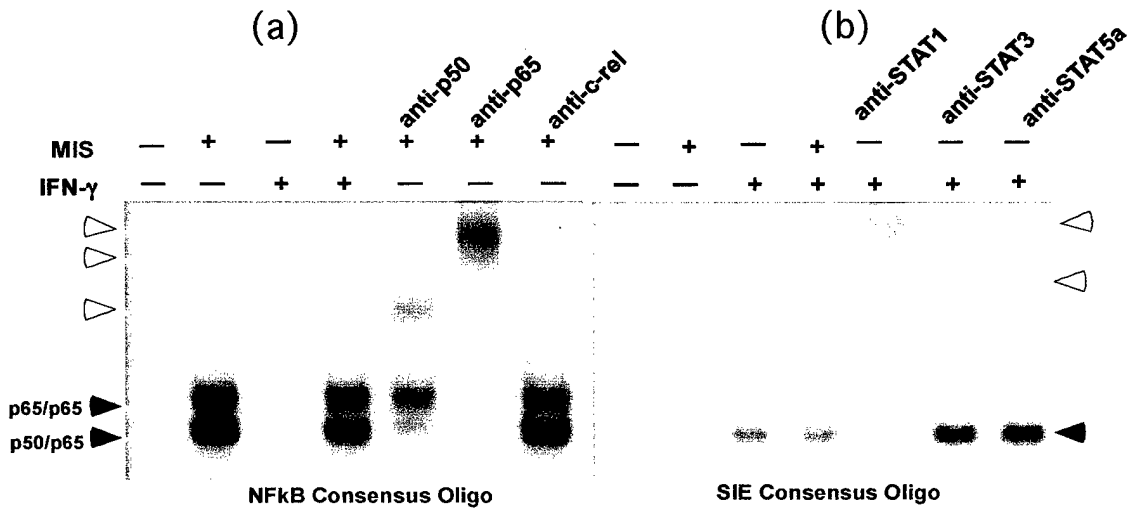


Fig. 1. T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ or both for 1 hour and 3 μg of nuclear proteins were analyzed by gelshift assay using 32P-labelled oligonucleotides containing the consensus DNA binding site for NFκB (a) or the STAT (b) proteins. (SIE: Stat Inducing Element)

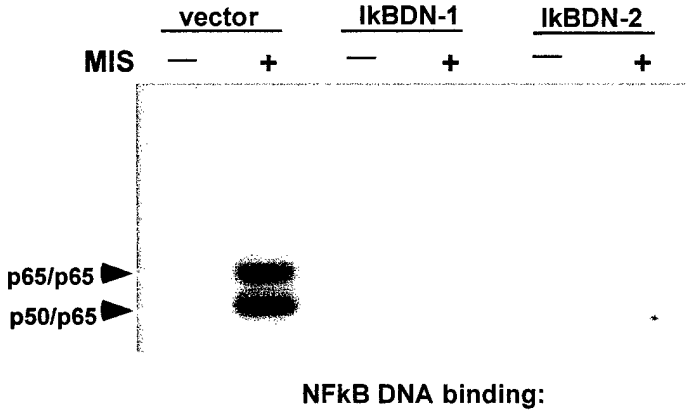


Fig. 2a. T47D cells stably transfected with either vector or IκBα-DN were treated with MIS for 0 and 2 hours and nuclear proteins were analyzed by gelshift assay to determine NFκB DNA binding activity.

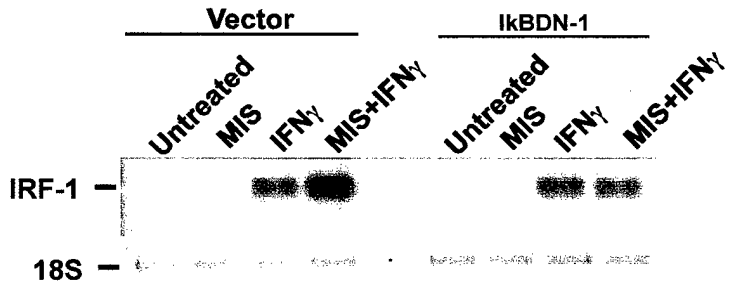


Fig. 2b. Vector and IκBα-DN-expressing T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ or both for 2 hours and total RNA (5μg) was analyzed for IRF-1 expression.

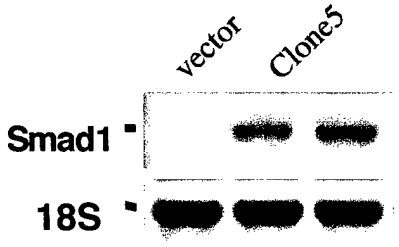


Fig. 3a. Expression of Smad1dN in T47D cells stably transfected with Smad1DN construct

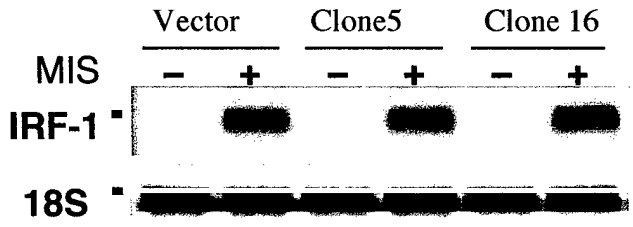


Fig. 3b Expression of IRF1 in vector transfected and Smad1DN was analysed by northern blot.

Fig. 4

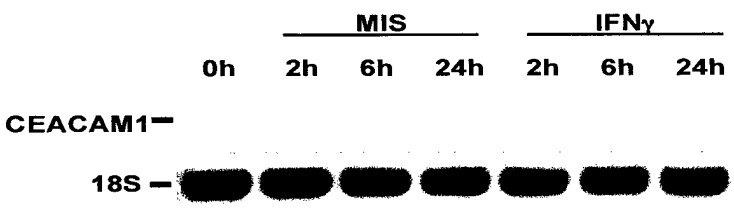


Fig. 4a. T47D cells were treated with 35 nM MIS or 1 ng/ml IFN-g for increasing periods of time. Total RNA was analysed for CEACAM1 expression by northern blot.

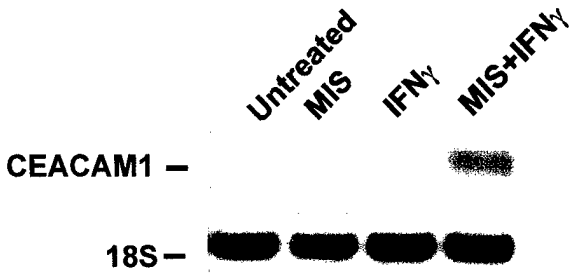
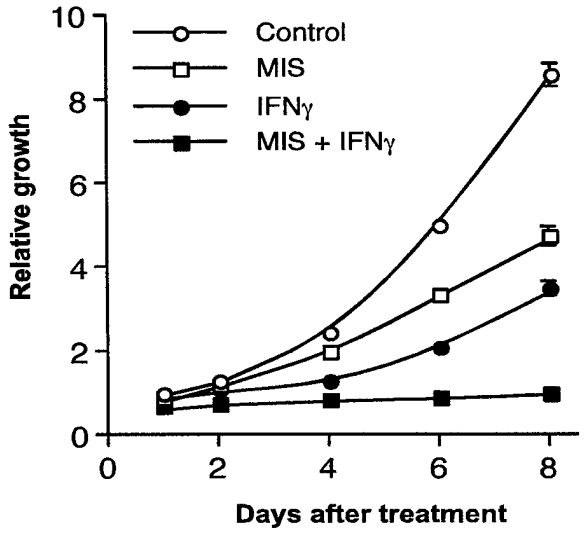


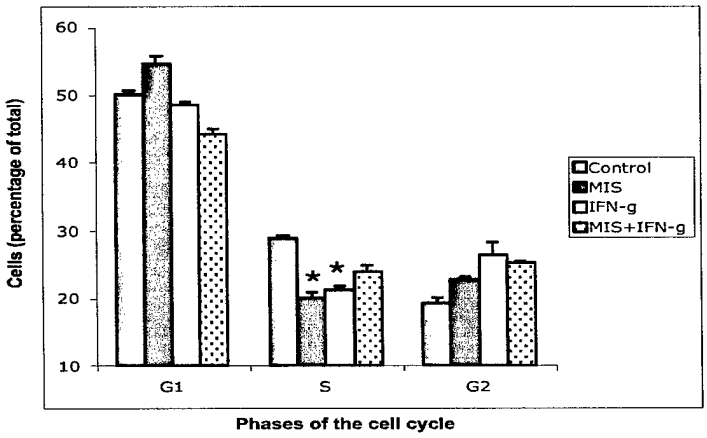
Fig. 4b. T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-g or both for 24 hours. Total RNA isolated from cells was analyzed for CEACAM1 expression. Hybridization to 18S rRNA is shown.

Fig. 5a



MIS and IFN-g were added at a concentration of 35nM and 5ng/ml, respectively, to MDA-MB-468 cells seeded in a 96 well plate. Cèll viability was determined after 1, 2, 4, 6 and 8 days by analysis of MTT conversion. Plates were anlyzed in an ELISA plate reader at 550 nm with a reference wave length of 630nm (n=8).

Fig. 5b



Cell cycle analysis of MDA-MB-468 cells treated with MIS and IFN-g. Cells were treated with 35nM MIS and 5ng/ml IFN-g or both for 72 hours. DNA content was analyzed by FACS. Statistical analysis was done using Student's t-test.

Key Accomplishments:

- MIS and IFN-γ function through distinct molecular pathways. MIS induced NFkB DNA binding activity in breast cancer cells where as IFN-γ functions through Stat1 DNA binding activity as is observed by Gel shift assays.
- Induction of IRF-1 by MIS was greatly reduced in the clone harboring IkBα-DN compared to cells transfected with the empty vector whereas IFN-γ does not induce IRF1 through NFkB. Co-stimulation of IRF-1 by MIS and IFN-γ in breast cancer cells is mediated through activation of NFkB and STAT pathways, respectively.
- MIS mediated induction of IRF-1 does not require phosphorylation of Smad1 as similar levels of IRF-1 induction are observed by MIS in vector and Smad1DN transfected T47D cells.
- Both MIS and IFN-γ induced CEACAM1 expression in T47D cells. Interestingly, simultaneous addition of MIS and IFN-γ resulted in synergistic induction of CEACAM1 expression in T47D cells.
- Since the signaling events initiated by MIS and IFN-γ converge to increase the magnitude of gene expression, we next tested their effect on the growth of breast cancer cells. Treatment of MDA-MB-468 cells with either MIS or IFN-γ inhibited growth and the presence of both inhibited growth better.
- Compared to untreated cells, MIS or IFN-γ treatment consistently led to a statistically significant decrease in the number of cells in the S-phase of the cell cycle ($p<0.001$ by Student's t-test). Interestingly, in cultures treated with a combination of MIS and IFN-γ, the percentage of cells in the S-phase did not demonstrate a greater decrease compared to that seen with either agent alone. Thus the enhanced inhibition of breast cancer cell growth by MIS and IFN-γ could not be explained by combined changes in cell cycle progression compared to treatment with either agent alone.

Currently, we are working on the effect of MIS and IFN-γ on the gene expression of lysyl oxidase, p21 and IFN-β. Also, the apoptosis assays will be carried out to test the pathway by which MIS and IFN-γ inhibit the breast cancer cell growth. The next important aspect of this project is to determine whether the synergistic interaction between MIS and IFN-γ to suppress breast cancer cell growth *in vitro* can be extrapolated to *in vivo* system. To test this we will be using various mouse models for the optimal tumor growth and the model with most robust growth will be used to test the effect of MIS and IFN-γ on the tumor regression.

Reportable outcomes:

Publications:

1. **V. Gupta**, D. P. Harkin, H. Kawakubo and S. Maheswaran. Transforming growth factor-β superfamily: Evaluation as breast cancer biomarkers and preventive agents. Current Cancer Drug Targets, 2004, 4, 1-11.

2. Y. Hoshiya, **V. Gupta**, H. Kawakubo, E. Brachtel, J.L.Carey, L.M.Sasur, A. Scott, P.K. Donahoe and S. Maheswaran. Mullerian inhibiting substance promotes interferon γ induced gene expression and apoptosis in breast cancer cells. The Journal of Biological Chemistry, 2003, 278, 51703-12.

Presentations

1. "MIS promotes interferon-γ mediated apoptosis of breast cancer cells". **V. Gupta**, Y. Hoshiya and S. Maheswaran at "Molecular Targets of Breast and Prostate Cancer" 2003 Joint retreat for Programs of Cell Biology, Breast cancer and Prostate cancer in the DF/ HCC.
2. "MIS promotes IFN-γ-induced gene expression and apoptosis in breast cancer cells. **Vandana Gupta**, Yasunori Hoshiya, Hirofumi Kawakubo, Elena Brachtel, Jennifer L. Carey, Laura Sasur, Andrew Scott, Patricia K. Donahoe and Shyamala Maheswaran at the SAC Poster 2003, MGH.
3. " Mullerian Inhibiting Substance promotes interferon induced IRF-1 expression and suppression of breast cancer cell growth" **V. Gupta**, Y. Hoshiya, P.K.Donahoe and S. Maheswaran at AACR, Feb. 2004 at Orlando, FL.

Conclusions:

1. MIS and IFN-γ function through distinct molecular pathways in breast cancer cells.
2. MIS and IFN-γ induce IRF1 expression in T47D cells through NFkB and STAT pathway repectively.
3. MIS mediated induction of IRF1 expression does not require Smad1 phosphorylation.
4. MIS and IFN-γ synergistically induce CEACAM1 expression in T47D cells.
5. MIS improves the growth inhibitory effect of IFN-γ in breast cancer cells.
6. The enhanced inhibition of breast cancer cell growth by MIS and IFN-γ does not involve changes in cell cycle progression.

Current Cancer Drug Targets, 2004, *4*, 165-182

Transforming Growth Factor-β Superfamily: Evaluation as Breast Cancer Biomarkers and Preventive Agents

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Abstract: The Transforming Growth Factor-β (TGFβ) superfamily of cytokines is comprised of a number of structurally-related, secreted polypeptides that regulate a multitude of cellular processes including proliferation, differentiation and neoplastic transformation. These growth regulatory molecules induce ligand-mediated hetero-oligomerization of distinct type II and type I serine/threonine kinase receptors that transmit signals predominantly through receptor-activated Smad proteins but also induce Smad-independent pathways. Ligands, receptors and intracellular mediators of signaling initiated by members of the TGFβ family are expressed in the mammary gland and disruption of these pathways may contribute to the development and progression of human breast cancer. Since many facets of TGFβ and breast cancer have been recently reviewed in several articles, except for discussion of recent developments on some aspects of TGFβ, the major focus of this review will be on the role of activins, inhibins, BMPs, nodal and MIS-signaling in breast cancer with emphasis on their utility as potential diagnostic, prognostic and therapeutic targets.

INTRODUCTION

Mammary development in humans and mice begins during the embryonic stage and at the end of intrauterine life the gland consists of a small number of branched ducts, which grow and branch at a very slow rate during prepubertal life. At puberty, ovarian hormones orchestrate the proliferation of stromal and epithelial tissue and the ducts elongate and sprout into an arborized structure with secondary branches. With the acquirement of sexual maturity, no further development of glandular or stromal components occurs until pregnancy [1]. During pregnancy, under the influence of luteal and placental sex steroids and other hormones, the epithelial cells undergo extensive proliferation resulting in a marked increase in ductular, lobular and alveolar growth. At the completion of lobuloalveolar growth, terminal differentiation of the secretory cells leads to synthesis and secretion of milk proteins. During weaning, the glandular epithelial cells of the mammary gland undergo extensive apoptosis, and the mammary gland returns to a histologic state similar to that which is observed in the postpubertal stage [2].

Mammary gland development requires tightly integrated regulation of growth, differentiation, apoptosis and extensive interaction between the epithelium and surrounding stroma, all of which are governed by a myriad of mammotrophic hormones and growth factors. During postnatal morphogenesis of the mammary gland, ductal elongation and branching appear to be under the positive influence of ovarian estrogen, progesterone, pituitary growth hormone and polypeptide growth factors including members of the epidermal growth factor family [3]. The stimulatory effects of these growth factors in the mammary gland are opposed by finely controlled factors, some of which are members of the TGFβ family that inhibit growth, induce differentiation and control the progression of cancer both *in vitro* and *in vivo* [4-14].

Alterations of normal growth-regulating mechanisms in the mammary gland lead to abnormal proliferation that contributes to an overall imbalance in growth. Pre-malignant breast lesions arise primarily from stem cells in the normal terminal duct lobular units. Invasive breast cancers arise from pre-existing premalignant breast lesions that include atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS and LCIS possess some malignant properties but lack the ability to invade and metastasize. Although the mechanisms that govern tumor progression remain unknown, as with the model proposed for colon cancer, breast cancer is thought to arise through the progression from atypical hyperplasia to *in situ* carcinoma to invasive breast cancer [15]. Comprehensive analysis of gene expression changes during disease progression and further delineation of pathways that govern growth regulatory processes in the mammary gland may be useful as prognostic and diagnostic markers of breast cancer development and provide novel targets for therapeutic intervention.

COMPONENTS OF THE TGFβ FAMILY SIGNALING CASCADE

The TGFβ superfamily consists of over 35 structurally and functionally related polypeptides including TGFβ, activins, inhibins, BMPs (bone morphogenetic proteins),

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GDFs (growth and differentiation factors), MIS (Mullerian Inhibiting Substance), Nodal and others that play an important role in the regulation of a multitude of diverse cellular processes. TGFβs, activins and MIS transduce their signals by binding to a type II receptor, a transmembrane serine-threonine kinase, that in turn complexes with another distinct transmembrane serine-threonine kinase known as the type I receptor. In contrast, some BMPs appear to bind directly to both type I and type II BMP receptors. Thus far in vertebrates five type II receptors and seven type I receptors have been identified indicating receptor promiscuity in order to accommodate all the ligands (Table 1A). Biological activity and signaling by ligands is also influenced by membrane-associated proteins such as endoglin, betaglycan and Cripto-1 and by extracellular proteins decorin, follistatin, noggin, chordin, Gremlin, DAN and cerberus [16-22].

Ligand-induced heteromeric complex formation of type I and type II receptors leads to phosphorylation of the type I receptor and induction of its latent kinase activity that subsequently propagates a signaling cascade that is mediated by phosphorylation of receptor activated Smad (R-Smad) proteins or through Smad-independent pathways. The R-Smad family is divided into ligand or receptor specific pathways; Smads1, -5 and -8 transmit BMP and MIS-stimulated signals and Smad2 and –3 mediate TGFβ and activin-induced signals. The phophorylated receptor specific R-Smads heteromerize with the common Smad4, translocate to the nucleus and associate with other transcriptional regulators to positively or negatively modulate transcription. Another class of Smads, Smad6 and –7 inhibit signaling by TGFβ family by either binding to R-Smad or by blocking their access to the type I receptors [20, 22, 23]. Although the major signaling route for the TGFβ family appears to be through receptor-activated Smad proteins, Smad-independent pathways constituted by protein kinases Akt, TAK1 (TGFβ-activated kinase) and their downstream effectors such as MAPK (mitogen-activated protein kinase) including ERKs (extracellular signal-regulated kinases), p38 and JNK (c-Jun-NH2-terminal kinase) are also activated following ligand binding [22, 23]. The NFkB (nuclear Factor kappaB) family of transcription factors, the expression and DNA binding activity of which is aberrantly regulated in many cancers including breast cancer is also regulated by TGFβ and MIS in human breast cancer cells [24, 25].

The role of TGFβ in development, differentiation, function and carcinogenesis of the mammary gland has been reviewed in several recently published articles [4-14]. Therefore, the main objective of this review is to evaluate the expression and functional significance of activins, inhibins, BMPs, nodal and MIS ligands, their receptors (Table 1) and extracellular and intracellular mediators of signaling in the developmental biology of the mammary gland. We will also describe the molecular aberrations in this pathway that may play a role in the development and progression of human breast cancer and assess their utility as potential diagnostic, prognostic and therapeutic targets.

ACTIVINS AND INHIBINS

Activins and inhibins are dimeric glycoproteins that consist of two common β subunits, βA and βB. Activins are dimers of β subunits: activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Inhibins are heterodimers consisting of an α subunit and either a βA subunit (inhibin A) or a βB subunit (inhibin B). The most recognized role for acitivins and inhibins is in the regulation of gonandal function [26]. However, they have also been implicated in control of cell growth and tumorigenesis [27-29].

Expression of Activins and Inhibins in the Normal Breast

In mice, the steady state level of activin βB mRNA is higher in the mammary gland than in most tissues and is comparable to that seen in the ovaries. By northern analysis, similar levels of activin βB transcript were detected in virgin mice, throughout pregnancy and during lactation [30]. Expression of activin βA, βB and inhibin α [31-36] and the activin type II receptor [32, 33, 37] has also been demonstrated in tissues and cell lines derived from normal or neoplastic human breast: by immunohistochemical analyses high α and low βA and βB subunit expression was detected in the ductal and lobular mammary epithelial cells regardless of the menstrual cycle, but not in myoepithelial cells [31]. In contrast others have shown by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) and immunoassays performed on immunoaffinity-purified human breast cells that βA expression was restricted to myoepithelial cells [32]. Thus the spatial and temporal patterns of inhibin and activin expression in the mammary gland during development and postnatal morphogenesis remain to be clearly defined.

Expression of Activins and Inhibins in Breast Cancer

Many groups have compared the expression of activin and inhibin in normal and neoplastic breast tissue. Immunostaining of normal and mildly neoplastic breast tissue detected moderate to high levels of α and lower levels of βA and βB expression while malignant lesions stained weakly for α and were negative for βA and βB expression [31]. However, quantification of inhibin α and βA mRNA expression in the breast by RT-fluorescent based kinetic PCR demonstrated that although no difference in expression was detectable between non-invasive tumor and normal breast tissue, βA expression was higher in invasive breast tumors when compared to normal breast and noninvasive tumor tissue [35]. This observation was confirmed by Reis *et al* who reported higher levels of βA mRNA in breast carcinoma than in normal tissue [36] suggesting that activation of βA expression in breast tumors might play a role in the transformation and progression of cancer. However, contradicting this hypothesis is the observation that Her2 negative cancers contain significantly higher amounts of activin βA mRNA compared to the Her2 positive cancers although the latter is more aggressive [35].

Consistent with increased activin A expression in breast tumors, βA expression has been demonstrated in many established human breast cancer cell lines [32]. Increase in inhibins in adenocarcinoma and lobular carcinoma compared to normal tissue has also been documented [34] and immune reactivity to activin A, inhibin A and inhibin B in cystic

deletions [57], a systematic analysis of breast tumor samples is needed to determine whether expression and genetic alterations of activin receptors contribute to breast cancer.

Functional Roles of Activins and Inhibins in the Mammary Gland

A role for activin in cell growth and morphogenesis of the mammary gland is suggested by its ability to inhibit hepatocyte growth factor and scatter factor-induced tubule formation by human mammary organoids *in vitro* [32]. The phenotype of female mice in which both alleles encoding activin βB were deleted suggests that βB is required for mammary gland function and differentiation. These mice cannot produce activin B, activin AB or inhibin B and were unable to nurse their pups since ductal elongation and alveolar morphogenesis were retarded. Thus βA cannot substitute for the functional effects of βB. Tissue transplantation experiments indicated that the mammary glands were dependent on the βB produced by the gland itself. Examination of the mammary glands of these mice revealed no discernable difference in structure between wild type and βB -/- mice at 4 weeks of age. After 3 months the ductal tree was less developed due to underdevelopment of the lobulo-alveolar structures and failure of ductal outgrowth compared to the wild type. During pregnancy, end buds persisted and the expansion was less than that observed in wild type mice. However, although βB deficient mice had less mammary tissue, the alveolar epithelial cells clearly exhibited a differentiated phenotype with milk protein gene transcription [30].

Elevated expression of activin in breast tumors and serum of breast cancer patients does not favor the hypothesis that activin may be an inhibitor of breast cancer cell growth. In fact exposure of primary mammary luminal and myoepithelial cells to activin A did not affect growth or cell cycle progression *in vitro* [32]. However, activin treatment has been shown to inhibit the growth of estrogen receptor positive human breast cancer cell lines MCF7 and T47D *in vitro* [32, 64]. Inhibition of MCF7 growth by activin resulted from an increase in the fraction of cells in the G1 phase of the cell cycle. The data currently available indicates that ActRIIA is expressed in breast cancer cell lines [32, 33] despite the lack of expression in primary mammary epithelial cells [32]. While it is possible that breast cancer cells may express higher levels of activin receptors, which render them responsive to activin, it would not explain why primary breast tumors do not respond to the increased levels of tumor-produced activin. A comprehensive analysis of activin receptor expression in breast cancer would help us decipher the discrepancy between the *in vitro* growth inhibitory effect of activin and the inability of primary breast tumors to respond to increased levels of activin produced by the tumor.

NODAL AND ITS COFACTOR CRIPTO-1

The ALK4 receptor in addition to mediating responses to activin and inhibin is also utilized by different signal transduction pathways including Cripto-1, an essential cofactor for Nodal, another TGFβ-related factor [65]. Cripto-1 is an EGF-CFC family member that contains a variant epidermal growth factor (EGF) domain and a cyteine-rich motif [66]. Cripto-1 does not directly interact with the EGF receptor, erb B-2, erb B-3 or erb B-4 [67, 68] or with ActRIIA or ActRIIB, but specifically interacts with the extracellular domain of ALK4 to activate Nodal-dependent transcription from a TGFβ-activin transcriptional response element containing reporter construct [58]. In addition to binding ALK4, Cripto-1 can also interact with the orphan receptor ALK7. While Nodal requires Cripto-1 to interact with ALK4, it binds directly to ALK7 [69]. ActRIIA and ActRIIB have also been implicated in mediating Nodal signaling [16, 70, 71].

In EpH-4, a mouse mammary epithelial cell line, ectopic expression of Nodal resulted in responsiveness to Cripto-1 and subsequent phosphorylation of Smad-2 through an ALK4 dependent mechanism. Nodal expression, as demonstrated by RT-PCR analysis, is extremely low in the mammary glands of virgin and lactating mice and is almost undetectable in pregnant and weaned animals. Cripto-1 expression is elevated during pregnancy and lactation but is low during involution [72, 73].

Cripto-1 plays a role in mammary gland development and increases in carcinogen-induced and spontaneous mouse mammary tumors. Expression of Cripto-1 is detected in 80% of human primary infiltrating carcinomas and in only 13% of uninvolved adjacent breast tissue suggesting an activation of Cripto-1 during neoplastic transformation of the breast, reviewed in [74]. However, little is known about the expression of Nodal, ALK4 and ALK7 in breast cancer specimens. Although the overlapping expression patterns exhibited by Nodal and Cripto-1 in the lactating mammary gland suggests that these two proteins may be functioning together, their putative roles in development of the mammary gland and breast cancer are not known.

BONE MORPHOGENETIC PROTEINS (BMPS)

BMPs regulate growth, differentiation and apoptosis in various cell types and play an important role in embryonal development and tissue morphogenesis, including bone and cartilage formation. The more than 30 members of the BMP family described include the BMPs, osteogenic proteins (OPs), GDFs and the cartilage-derived morphogeneric proteins (CDMPs) [20, 23]. BMP type I (BMPRI) and type II (BMPRII) receptors bind BMPs with high affinity when expressed together and with lower affinity when expressed separately. BMPRII heteromerizes with ALK2, ALK3 also known as BMPRIA or ALK6 also known as BMPRIB (Fig (**1B**)). In addition to BMPRII, ActRIIA and ActRIIB also bind BMPs cooperatively when BMPRIA (ALK3) or BMPRIB (ALK6) is coexpressed in the cell [20, 22, 23].

Expression of BMPs and BMP Receptors in Normal and Neoplastic Breast Tissue

Of the many members of the BMP family, expression of BMP-2, -3, -4, -5, and -6 has been detected in various tissue and cell line derivatives of the breast [75-79]. BMP-2 and BMP-4 are expressed during both fetal and postnatal mammary gland development. Expression of BMP-2 can be

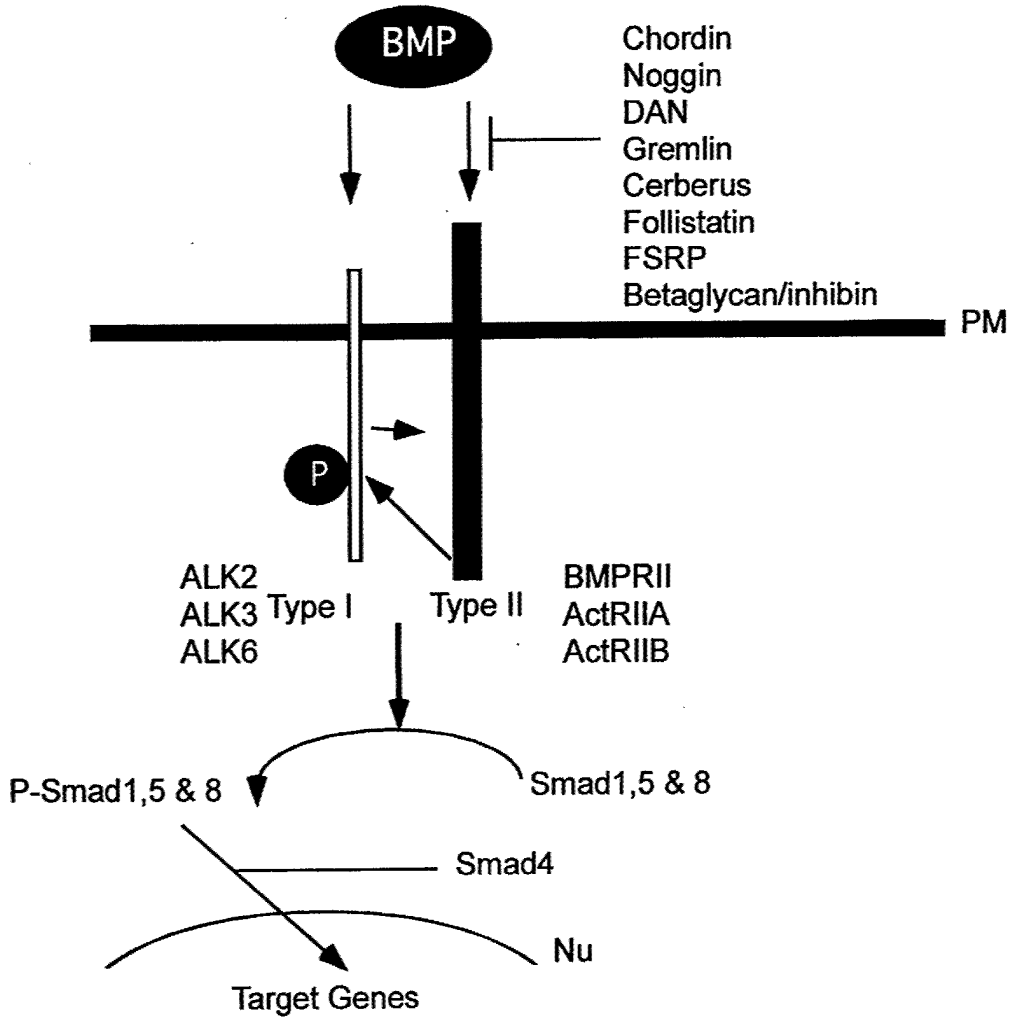

Fig. (1B). BMPs bind to BMP type I and type II receptors with high affinity when expressed together and with low affinity when expressed separately. In addition to BMPRII, ActRIIA and ActRIIB also bind BMPs when ALK3 or ALK6 are co-expressed. The extracellular proteins chordin, noggin, DAN, Gremlin, Cerberus, Follistatin and FSRP bind BMPs and antagonize BMP-signaling. The inhibin/betaglycan complex competes with BMPs for ActRII and BMPRII receptors and thus blocks cellular responses to BMPs. Propagation of BMP signals results in the activation of Smads 1, 5 and 8.
PM: Plasma memberane, Nu: Nucleus

BMP-6 is expressed in both estrogen receptor positive and negative breast cancer cell lines and in normal breast and breast tumor samples. Its expression in breast cancer cells is suppressed following serum starvation. EGF and EGF-like growth factors such as TGFα, amphiregulin, and betacellulin which positively regulate growth, elevate BMP-6 expression in breast cancer cells indicating that it may also positively regulate growth. However, analysis of BMP-6 mRNA in breast tumors demonstrated that the abundance of BMP-6 mRNA varies in breast tumors. Of 44 breast cancer patients tested, BMP-6 expression was down-regulated in the tumors of 18 patients (41%) compared to the tumor free margins while it was higher in tumor vs non-tumor samples in just 8 (18%) patients. In twelve of the 18 patients who had decreased BMP-6 expression in the tumor tissue, EGF receptor mRNA was found to be lower in the tumor compared to the matched normal tissue. The 8 patients with increased tumoral expression of BMP-6 did not express lower levels of EGF receptor compared to non-tumor tissue [78]. Thus the association between EGF and BMP-6 expression during mammary gland development and neoplastic progression and the functional interaction between EGF and BMP-6 merit further investigation.

BMP-6 expression in tumors has been implicated in bone metastasis. Prostate cancer patients with positive bone scan had detectable expression of BMP-6 compared to those with a negative bone scan [81]. Tateyama *et al* have demonstrated expression of BMP-6 in myoepithelial cells in canine mammary gland tumors. BMP-6 immunopositive proliferating foci had a mucinous stroma with marked hyaline and chondroid changes and BMP-6 negativity was associated with a lack of chondroid change [82]. BMPs secreted by breast cancer cells into the culture medium can upregulate the expression of bone sialoprotein in preosteoblast cells [76]. Bone metastasis is frequently observed during advanced stages of breast cancer resulting in local bone resorption [83]. Whether BMP-6 expression in

withdrawal correlated with an increase in BMP-7 mRNA [88]. Estrogens also reverse antiestrogen-induced BMP-4 promoter activity that is mediated through estrogen receptor α [89].

In addition to inhibiting estradiol-induced growth of breast cancer cells, BMP-2 can also inhibit the growth of estrogen receptor negative breast cancer cells. Arnold *et al* [75] have demonstrated that the estrogen receptor negative MDA-MB-231 cells are more responsive to the growth inhibitory effect of BMP-2 than the estrogen receptor positive MCF7 cells. As with MCF7 cells, BMP-2-mediated inhibition of MDA-MB-231 cells was associated with increased p21 expression and prevention of cyclin E and cyclin D1 associated kinase activity [90]. In MCF7 cells, BMP-2 also increases the expression of the helix-loop-helix proteins Id-1, Id-2 and Id-3 [77]. The molecular cross-talk between estrogen and BMPs and the estrogen-independent effects of BMPs on breast cancer cell growth is a subject that requires further investigation.

MULLERIAN INHIBITING SUBSTANCE (MIS)

Expression of MIS and MIS Receptors in Normal and Neoplastic Breast Tissue

MIS, a distant member of the TGFβ superfamily demonstrates a sexually dimorphic pattern of synthesis. It is secreted by Sertoli cells of the fetal and adult testis and by granulosa cells of the postnatal ovary. The most well known developmental role for MIS lies in the regression of the Mullerian duct, the anlagen of the uterus, Fallopian tubes and the upper part of the vagina. However, the presence of MIS in the adult serum even after regression of the Mullerian duct suggests a postnatal role for this gonadal hormone. Intracellular signaling by MIS is propagated following association of the ligand with the MIS type II receptor, a serine, threonine kinase highly homologous to the type II receptors of the TGFβ superfamily [91]. Ensuing signals are dependent on recruitment of a type I receptor. ALK2, ALK3 and ALK6 have been implicated in mediating MIS signaling in cells [92-96] (Fig (**1C**)).

MIS type II receptor mRNA was detected in normal mouse and rat breast by RNase protection assay. Analysis of MIS type II receptor mRNA levels in mammary glands during breast development in Sprague-Dawley rats revealed a gradual increase until postnatal day 30 and a decrease in three individual animals older than 30 days. Quantification of transcript levels by phophorimaging analysis demonstrated a 2.5 fold decrease in MIS type II receptor between animals of postnatal day 14-30 and postnatal day 40-60 [97]. Interestingly, the lowering of MIS type II receptor expression during mammary gland development in the rat coincided with puberty when the ductal system branches and invades the fat pad. Analysis of mammary glands of virgin, pregnant, lactating, and weaned rats demonstrated an 80% decrease in MIS type II receptor expression 2 days after delivery during early lactation. The receptor mRNA rebounded to higher levels two days after removal of pups, a period of ductal regression [97]. The inverse correlation between MIS type II receptor expression and growth in the breast during puberty and peripartum stages suggests that MIS-mediated signaling may exert an inhibitory effect on proliferation.

RNAse protection assays, northern blot analysis and RT-PCR using MIS type II receptor specific primers followed by sequence analysis demonstrated the presence of MIS type II receptor mRNA in human breast cancer cell lines [97]. Immunoblotting with the MIS type II receptor antibody confirmed the expression of MIS type II receptor protein in breast cancer cells. PCR amplification of cDNAs derived from the normal breast, nontumorigenic mammary epithelial cell lines (MCF10A and 184A1), breast fibroadenoma and invasive ductal carcinomas also revealed expression of the MIS type II receptor mRNA [25, 97]. Thus the mammary gland might be an additional target for the ovarian hormone MIS. However, local production of MIS was not detected in the mammary gland. Expression of MIS ligand was not detected by MIS-ELISA (Enzyme-linked immuno-sorbant assay) in culture supernatants obtained from either normal or cancer cell lines of the human breast or by RT-PCR analysis of cDNA derived from normal and tumor tissue obtained from human breast cancer patients [25, 97]. Thus, the mammary gland may be dependent on the MIS present in the serum.

MIS Inhibits Breast Cancer Cell Growth

The functional properties of MIS type II receptor expression in breast tissue was determined by assaying whether MIS alters the growth properties of the estrogen receptor positive breast cancer cell line T47D. Colony inhibition assays performed by cotransfection of MIS and the hygromycin resistance gene into T47D cells resulted in a 75% reduction of drug resistant colonies compared to vector transfected cells. The noncleavable inactive form of MIS did not affect the growth of T47D cells. Addition of exogenous MIS to proliferating T47D cells inhibited growth by increasing the fraction of cells in the G1 phase of the cell cycle compared to untreated cells. Activation of caspase-3, an enzyme activated in cells undergoing apoptosis, demonstrated that MIS induced a 3-fold induction in caspase-3 activity in T47D cells compared to untreated cells. Thus MIS inhibited the growth of breast cancer cell lines *in vitro* by interfering with cell cycle progression and inducing apoptosis. As with T47D cells, treatment of MDA-MB-231 cells with exogenous MIS inhibited growth by 50% [25].

Mammary tissue isolated from 6 week old RAG-2 (recombination activating gene-2) null female mice [98] injected with MIS had increased number of apoptotic cells compared to phosphate buffered saline treated control animals. The ratio of apoptotic cells per duct, normalized to controls, increased 8-fold in mammary epithelial cells exposed to MIS treatment compared to animals injected with vehicle control [97]. These results indicate that MIS, as with other members of the TGFβ superfamily may be useful in breast cancer therapy. To this end, its ability to regulate the growth of human breast cancer cell xenografts grown in immunosuppressed mice and its effect on mouse models of spontaneous mammary carcinoma are currently being investigated (Maheswaran *et al.* unpublished).

expression showed a significant correlation with overall and disease free survival [120, 121]. Furthermore, the serum levels of endoglin were higher in breast cancer patients compared to normal controls and significantly higher in patients who developed distant metastasis compared to disease-free patients [4, 122]. Measurement of endoglin-TGFβ1 complexes in the plasma of patients with breast cancer demonstrated elevated levels compared to that observed in age-matched normal women [4, 123]. In another study, measurement of TGFβ3 and TGFβ3-endoglin in 80 breast cancer patients demonstrated a significant elevation in the plasma of patients with positive lymph node metastasis compared to those without node metastasis. The levels of both TGFβ3 and TGFβ3-endoglin correlated with lymph node status although no significant correlation was seen between TGFβ3 and TGFβ3-endoglin and tumor stage, size or histological grade [124]. These results taken together suggest that measurement of endoglin-TGFβ complexes in the circulation and monitoring the overexpresion of endoglin in vascular endothelial cells may be of potential diagnostic and prognostic value in breast cancer.

An antibody against endoglin (TEC-11) when coupled to deglycosylated ricin A chain (dgA), potently inhibited protein synthesis in proliferating human umbilical vein endothelial cells *in vitro* compared to that observed in confluent cultures [115]. *In vivo*, a monoclonal antibody against human endoglin (K4-2C10) with weak reactivity against mouse endothelial cells, when coupled with dgA, suppressed the growth of MCF7 cell xenografts in immunosuppressed mice, an effect connected with the inhibition of tumor-associated blood vessels and disruption of tumor-associated angiogenesis. The unconjugated antibody was not significantly effective in the inhibition of tumor growth and importantly the immunotoxin did not produce any side effects in this mouse model system [116]. The regression of tumors was long lasting in a majority of MCF cell tumor bearing mice when administration of the conjugated antibody was limited to 3 times via the tail vein [125]. A similar experiment performed with ^{125}I-labeled anti-endoglin monoclonal antibodies SN6f and SN6j demonstrated that intravenous administration of the antibody had significant anti-tumor activity against tumors of MCF7 cells in SCID mice. As with the previous study, no toxicity, weight loss or significant organ damage was observed following the anti-angiogenic radiotherapy with ^{125}I-labeled SN6f or SN6j [126]. Thus anti-endoglin antibody coupled immunotoxins such as TEC-11-dgA and K4-2C10-dgA or radioimmunotherapy with anti-endoglin antibodies may have therapeutic value in the treatment of solid tumors including breast cancer by selectively killing vascular endothelial cells. Furthermore, imaging of spontaneous mammary adenocarcinomas in two dogs using an ^{125}I-anti-endoglin antibody [119] demonstrates that it may also represent a method for *in vivo* imaging of solid malignancies including breast cancer.

The levels of endoglin-TGFβ1 and TGFβ1 in pre-treatment plasma may also be of clinical value in identifying patients at risk of developing post-radiotherapy fibrosis. Fibrosis represents a serious complication associated with radiotherapy treatment of cancer patients. Quantification of TGFβ1 and endoglin-TGFβ1 complex in plasma samples obtained from 91 pre-radiotherapy, early stage breast cancer patients demonstrated that development of moderate to severe fibrosis was associated with significantly elevated levels of TGFβ1 and significantly lower levels of endoglin-TGFβ1 complex compared to those with no fibrosis [127].

Follistatin and FSRP

An ELISA to detect dimeric activin and follistatin demonstrated the presence of both in human milk. Although no significant difference in concentration was evident between the third and fifth day of delivery, or between spontaneous delivery and cesarean section, a significant decrease was observed after one month of lactation [128].

Many studies including mice that are homozygously deleted for the inhibin alpha allele and carry the mouse metallothionine I follistatin transgene indicate a role for follistatin in tumor growth [129]. Comparison of protein maps of normal and malignant prostate demonstrated the loss of a follistatin-related protein during malignant transformation [130]. Follistatin levels are also elevated in patients with hepatocellular carcinoma compared to those of controls [131] and increased levels of follistatin mRNA has been demonstrated in murine hepatocellular carcinomas when compared to the matched normal tissue [132]. In addition, Wnt3a, a developmentally regulated signaling molecule that induces the transformation and changes in morphology of mammary cells [133], robustly induces follistatin [134].

The currently available data strongly suggest a role for activin mediated signaling in development, morphogenesis and neoplastic tranformation of the mammary gland. Since follistatin expression determines the relative concentration of biologically functional activin A within a specific microenvironment, studies that correlate follistatin and activin expression in the developing breast and breast cancer will help define their potential utility as diagnostic and prognostic targets in breast cancer.

Antagonists of BMP Signals

Expression analyses of BMPs in breast cancer suggests that local concentrations of these proteins may be important in rendering proper regulation of mammary epithelial cell proliferation and may play a role in breast cancer progression. Although the role of these proteins in governing developmental processes is an area of active research, their function in neoplastic transformation and cancer has not been well studied.

Loss of heterozygozity on the short arm of chromosome 1 has been reported in many studies involving breast cancer [135-138] and the DAN gene maps to human chromosome 1p36.11-1p36.13 [35]. Bieche *et al* 1999, recently identified 1p36.3 and 1p32, as two distinct regions showing much higher levels of LOH within the high background of LOH throughout 1p32-pter [139]. This study together with the publication of transcript maps of the human genome ruled out the presence of several candidate genes for breast cancer including DAN. However, electronic profiling of the prostate expressed sequence tags and RT-PCR analysis of prostate cancers of Gleason score 6 demonstrated that expression of DAN was reduced by 80% in cancer compared with normal

have phospho-Smad2 expression in the tumor cells. The loss of Smad2 phosphorylation in these specimens was not due to structural abrnormalities in the C-terminal phosphorylation site of the Smad2 gene. Although loss of TGFβ type II receptor expression has been detected in a subset of breast cancer with aggressive phenotype [149, 150], loss of phosphorylated Smad2 in these tumors was not attributable to mutations in either TGFβ type I or type II receptors. Loss of Smad 4 expression was seen in 1.3% of the tumors tested while 3 cases failed to have either phosphorylated Smad2 or Smad4. Absence of phospho-Smad2 or Smad4 did not correlate with tumor histological type, tumor grade, nuclear grade, estrogen-, progesterone-, and HER2/neu-receptor expression or lymph node status.

Although lack of phospho-Smad2 does not seem to correlate with prognostic and predictive parameters, the overall survival of patients whose cancers failed to express phopho-Smad2 was significantly shorter than those with phosho-Smad2 expression. Thus absence of phospho-Smad2 expression may define a subset of aggressive breast cancers and may represent a novel predictor of poor outcome. A similar association between absence of phospho-Smad2 and poor survival was also observed in patients with sporadic colorectal cancer [151, 152]. These observations suggest that in addition to the latent form of TGFβ that is deposited in the extracellular matrix, the mammary gland may also contain biologically active TGFβ. Alternatively, the detection of dimeric activin in homogenates of breast tissue [36] suggests that phosphorylation of Smad2 may be elicited by paracrine/autocrine action of activin although it is surprising that the higher levels of activin A detected in homogenates of breast cancer tissue compared to non-neoplastic tissue [36] is not reflected in the levels of phospho-Smad2 observed in tumor vs normal breast tissue.

Role of Smads in Bone Metastasis of Breast Cancer

Breast cancer commonly metastasizes to the bone in patients with advanced disease and causes bone destruction. Parathyroid hormone-related protein (PTHrP), a tumorally expressed factor is responsible for bone destruction associated with breast cancer. The MDA-MB-231 cell line that produces moderate amounts of PTHrP has been used extensively to investigate metastasis of breast cancer cells to the bone [153-155]. In MDA-MB-231 cells, overexpression of wild type Smad2, -3 and-4 increased the induction of PTHrP by TGFβ, [154]. PTHrP is one of the most abundant growth factors released in active form into the bone microenvironment as a result of osteoclastic bone resorption. Dominant negative Smad2, Smad3 or Smad4 paritally reduced TGFβ-mediated stimulation of PTHrP in these cells [154]. Compared to Smad3 alone, Smad3 along with Ets1 strongly activates the P3 promoter of the PTHrP gene in MDA-MD-231 cells [155]. In addition to Smads, activation of PTHrP by TGFβ is also regulated by Smad-independent activation of the p38 MAP kinase pathway [154].

FUTURE PERSPECTIVES

A review of the available data strongly suggests that TGFβ family ligands, their regulatory proteins and mediators of signaling play important roles in the complex processes that govern mammary gland development, neoplastic transformation and progression of breast cancer. While a plethora of studies have addressed the importance of TGFβ in these events, the functional significance of activins, inhibins, MIS and BMPs in development and carcinogenesis of the breast is just beginning to emerge. Elucidation of the function of activin, which is overexpressed in breast tumors yet inhibits the growth of breast cancer cells *in vitro*, in conjunction with its extracellular regulator follistatin and FSRP, of which very little data is available on mammary gland development and breast cancer, is an area that needs further investigation. Lowering of BMP expression in breast tumors and studies that demonstrate BMP-mediated inhibition of breast cancer cell growth *in vitro*, strongly support the hypothesis that BMPs may be growth regulators in the mammary gland. The prognostic, diagnostic and therapeutic importance of these observations remains to be evaluated. The presence of receptors that bind MIS and its ability to induce apoptosis in breast cancer cells *in vitro* and in mammary epithelial cells *in vivo* argues that it may also be an endogenous regulator of growth. The therapeutic utility of MIS in treatment of breast cancer is currently being evaluated in mouse models with spontaneous mammary carcinoma and in immunosuppressed mice bearing human breast cancer cell xenografts (Maheswaran *et al.* unpublished).

Elucidating the specific role of each TGFβ-like ligand in development, differentiation, transformation and tumor progression of the mammary gland is complicated by the promiscuity of components involved in this signaling cascade. An extensive analysis of the interplay between the various ligands and identification of the role of signaling intermediates during these processes, would pave the way for mechanism-based design of anti-cancer drugs, which would be beneficial in the treatment of patients with breast cancer.

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ABBREVIATIONS

DCIS	=	Ductal Carcinoma In Situ
LCIS	=	Lobular Carcinoma In Situ
BMP	=	Bone Morphogenetic Protein
GDF	=	Growth and Differentiation Protein
MIS	=	Mullerian Inhibiting Substance
R-Smad	=	Receptor-Activated Smad
HCG	=	Human Chorionic Gonadotropin
HBEC	=	Human Breast Epithelial Cell
InhBP	=	Inhibin Binding Protein

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Mullerian Inhibiting Substance Promotes Interferon γ-induced Gene Expression and Apoptosis in Breast Cancer Cells*

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This report demonstrates that in addition to interferons and cytokines, members of the TGFβ superfamily such as Mullerian inhibiting substance (MIS) and activin A also regulate IRF-1 expression. MIS induced IRF-1 expression in the mammary glands of mice *in vivo* and in breast cancer cells *in vitro* and stimulation of IRF-1 by MIS was dependent on activation of the NFκB pathway. In the rat mammary gland, IRF-1 expression gradually decreased during pregnancy and lactation but increased at involution. In breast cancer, the IRF-1 protein was absent in 13% of tumors tested compared with matched normal glands. Consistent with its growth suppressive activity, expression of IRF-1 in breast cancer cells induced apoptosis. Treatment of breast cancer cells with MIS and interferon γ (IFN-γ) co-stimulated IRF-1 and CEACAM1 expression and synergistic induction of CEACAM1 by a combination of MIS and IFN-γ was impaired by antisense IRF-1 expression. Furthermore, a combination of IFN-γ and MIS inhibited the growth of breast cancer cells to a greater extent than either one alone. Both reagents alone significantly decreased the fraction of cells in the S-phase of the cell cycle, an effect not enhanced when they were used in combination. However, MIS promoted IFN-γ-induced apoptosis demonstrating a functional interaction between these two classes of signaling molecules in regulation of breast cancer cell growth.

Mullerian Inhibiting Substance (MIS)[1] is a member of the TGFβ family, a class of molecules that regulates growth, differentiation, and apoptosis in many cell types. In the male embryo, MIS causes regression of the Mullerian duct, the anlagen of the Fallopian tubes, uterus, and the upper vagina (1). However, a postnatal role for MIS in males and females has yet to be clearly defined. MIS receptor mRNA in the mammary gland significantly diminishes during puberty when the ductal system branches and invades the adipose stroma and during the expansive growth at pregnancy and lactation, but is upregulated during involution, a time of regression and apoptosis (2, 3). The inverse correlation between MIS type II receptor expression and various stages of mammary growth suggests that MIS-mediated signaling may exert an inhibitory effect on mammary gland growth. Consistent with this concept, MIS inhibited the growth of both estrogen receptor (ER)-positive and -negative breast cancer cells by inducing cell cycle arrest and apoptosis (4).

Type I (IFN-α and IFN-β) and type II (IFN-γ) interferons are a family of antiviral cytokines that exhibit immunomodulatory and anti-proliferative effects (5). The antitumor effects of cytokines such as interleukin-12, in murine mammary carcinogenesis models correlate with high levels of serum IFN-γ (6–12). IFN-γ induced tumor regression results from immune surveillance of tumor cells and from direct cytotoxic effects (13–17), which are evident from its ability to inhibit the growth of several tumor-derived cell lines (18, 19) including breast cancer cells (20–22). Intralesional injections of IFN-α and IFN-γ into breast cancer patients with skin recurrences resulted in either complete or partial regression of the skin lesions but was associated with clinical toxicity in all patients (23). Thus identification of molecules that enhance the antitumor effects of IFN-γ may render it effective at lower doses, reduce clinical toxicity associated with high concentrations of the drug, and expand their therapeutic applications.

IFN-γ-induced growth inhibition requires coordinate expression of specific genes. Interferon regulatory factor-1 (IRF-1) is robustly induced by both type I and type II interferons. In addition to its important role in innate and adaptive immunity (24), IRF-1 also plays a role in regulating the growth of different mammalian cell lines (25). Different aspects of the tumor suppressor function of IRF-1 may be explained, at least in part, by the observation that it induces several growth regulatory genes including those with anti-proliferative activity such as IFNα/β, p21, and the cell adhesion molecule CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule) (25).

Using DNA microarrays to profile gene expression, we identified that treatment of breast cancer cells with MIS strongly induces the expression of IRF-1. Since interferons also strongly induce IRF-1 (15), we tested whether intersection of MIS and

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[1] The abbreviations used are: MIS, Mullerian inhibiting substance; IRF, interferon regulatory factor-1; IFN, interferon; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STAT, signal transducer and activator of transcription; FACS, fluorescence-activated cell sorter; DAPI, 4′,6-diamidino-2-phenylindole; SIE, stat-inducing element; FITC, fluorescein isothiocyanate; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule; EST, expressed sequence tag; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

IFN-γ signaling pathways would result in enhanced expression of downstream target genes and increased antiproliferative activity against breast cancer cells. In this report, we demonstrate that members of the TGFβ superfamily including MIS induce IRF-1 expression in immortalized human mammary epithelial cells with characteristics of normal cells. Treatment of breast cancer cells with MIS and IFN-γ led to synergistic induction of CEACAM-1 through an IRF-1-dependent mechanism. Furthermore, a combination of MIS and IFN-γ led to a greater degree of growth inhibition compared with either agent alone due to enhanced apoptosis rather than a combinatorial effect on cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell Culture and MTT Assays—Human breast cancer cell lines T47D and MDA-MB-468 were grown in Dulbecco's modified medium supplemented with 10% female fetal bovine serum, glutamine, and penicillin/streptomycin. The human mammary epithelial cell line MCF10A was grown in mammary epithelial growth medium (MEGM, Clonetics) supplemented with 100 ng/ml of cholera toxin (Calbiochem). Human recombinant MIS (26) was collected from growth media of Chinese hamster ovary cells transfected with the human MIS gene and purified as described (26). Recombinant human IFN-γ was purchased from Sigma and IFN-β and activin A were from R&D systems, Inc. TGFβ was a kind gift from Dr. Anita Roberts.

Estimation of cell growth was based on the colorimetric reduction of a yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), to a purple formazan by viable cells. The MDA-MB-468 cell suspension (3000 cells/well) was transferred to a 96-well microtiter plate. MIS, IFN-γ, or both were added to the wells once on day 0 at concentrations indicated in the figure legends. The number of viable cells was estimated by adding 30 μl of MTT solution (5 mg/ml in phosphate-buffered saline). Following 3 h of incubation at 37 °C, the stain was eluted into 200 μl of Me_2SO. The optical densities were quantified at a test wavelength of 550 nm and a reference wavelength of 630 nm on a multiwell spectrophotometer. Statistical analysis was done using Student's *t* test.

T47D cells expressing antisense IRF-1 mRNA were generated by co-transfecting 0.5 μg of hygromycin resistance plasmid and 10 μg of pCDNA 3.1 expressing the IRF-1 transcript in the antisense orientation. Cells were maintained in medium containing 100 μg/ml of hygromycin (Roche Applied Science), and clones expressing antisense IRF-1 were identified by Northern blot analysis.

Western Blot Analysis—Expression of protein in cells was analyzed by Western blot using the rabbit anti-IRF-1 (Santa Cruz Biotechnology), rabbit antiphospho-STAT1, and rabbit anti-STAT1 (Cell Signaling) antibodies according to the protocol described (27). Nuclear and cytoplasmic protein fractions from T47D cells were isolated according to the protocol described below.

NFκB and STAT Electrophoretic Mobility Shift Assays—T47D cells were grown to 70% confluence and treated with indicated concentrations of rhMIS or IFN-γ. Cells were harvested in cold PBS, resuspended in 1 ml TKM 10:10:1 (10 mM Tris, pH 8.0, 10 mM KCl, and 1 mM $MgCl_2$) and lysed with 0.1% Triton X-100. The cytoplasmic fraction was stored frozen for Western blot analysis of STAT proteins. Nuclei were pelleted by centrifugation at 5,000 rpm at 4 °C, and proteins were extracted in buffer containing 10 mM HEPES pH 7.0, 350 mM NaCl, and 1 mM EDTA. 3 μg of protein were used in 25-μl binding reactions containing 10 mM HEPES pH 7.0, 70 mM NaCl, 0.1% Triton X-100, and 4% glycerol. NFκB (Promega), and SIE (Stat Inducing Element; Geneka) oligonucleotides were 5′-end-labeled with ^{32}P and DNA protein complexes were resolved on 4% native polyacrylamide gels. Supershift experiments were performed by adding 1 μg of rabbit anti-p65 or p50 antibodies (Santa Cruz Biotechnology) or rabbit anti-STAT1, STAT3, or STAT5α (Santa Cruz Biotechnology) antibodies to the binding reactions.

Animals and MIS Treatment—All animals were cared for and experiments were performed under AAALAS approved guidelines using protocols approved by the Institutional Review Board-Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Ketamine/xylazine (100/10 mg/kg) was used for anesthesia. To study the effect of rhMIS on the mammary gland, adult female C3H mice (8-week-old; average weight 25 grams) were obtained from the Edwin L. Steele Laboratory, Massachusetts General Hospital, Boston, MA. Each animal was injected intraperitoneally with 100 μg of rhMIS or phosphate-buffered saline (vehicle control). Breast tissue was harvested bilaterally from each animal for RNA isolation. Blood was drawn from the animals at the time of tissue harvest to determine the circulating level of rhMIS using MIS-ELISA.

IRF-1 expression analysis in the rat breast during perinatal morphogenesis was done using Sprague-Dawley rats. To analyze expression during lactation and involution, after the pups were born (postdelivery), some animals were housed with the pups (lactating) while others were weaned 2 days after lactation (weaned).

RNA Analysis—Total RNA from T47 cells treated with MIS for 0 and 1 h was isolated using RNA STAT-60 and sent to the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital for profiling gene expression using HG-U95Av2 oligonucleotide arrays (Affymetrix) containing ~12,500 full-length annotated genes together with additional probe sets designed to represent EST sequences. The EST clones to detect the expression of IRF-1 and CEACAM1 were purchased from Incyte Genomics Inc. For Northern blot analysis, equal amounts of RNA were separated on a formaldehyde gel, transferred to Hybond-N membrane (Amersham Biosciences) and probed with either IRF-1 or CEACAM1.

Cell Cycle Analysis and Apoptosis Assays—The cell cycle distribution of untreated and MIS and IFN-γ-treated cultures was analyzed by fluorescence activated cell sorting (FACS). Cells were detached with PBS/EDTA, fixed in 95% ethanol and treated with propidium iodide and RNase A. Flow cytometric analysis was performed on a Becton Dickinson FACScan flow cytometer.

To measure the apoptosis, cells treated with MIS and IFN-γ were immunostained with a FITC-conjugated anti-annexin V antibody, counterstained with DAPI and analyzed by FACS. The effect of IRF-1 on apoptosis, was analyzed by transiently transfecting cells in logarithmic growth phase with either the empty vector (4 μg) or IRF-1 (4 μg), along with a plasmid encoding the cell surface marker CD20 (1 μg) as described in (28). Cells were harvested 72 h after transfection and stained for CD20 and annexin V. Apoptosis in CD20-positive cells was determined by FACS analysis.

Immunohistochemical Analysis of IRF-1—Twenty-three cases of human breast carcinoma with adjacent uninvolved breast parenchyma were selected from the files of the Massachusetts General Hospital (MGH) Pathology Department according to protocols approved by the Human Research Committee at MGH. These included 13 poorly differentiated (histologic grade 3/3), 5 moderately differentiated (grade 2/3) and 3 well-differentiated (grade 1/3) ductal adenocarcinomas. Patient age ranged from 34 to 81 years at the time of the procedure. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections according to standard procedures. Deparaffinized tissue sections were treated with 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase. After the sections had been microwaved in a 10 mM citrate buffer solution at 100 watts for 25 min, they were incubated with 2% normal goat serum for 30 min to block any nonspecific protein binding. The sections were incubated with rabbit antibodies against IRF-1 (Santa Cruz Biotechnology) at 4 °C overnight at a 1:400 dilution. After incubation with biotinylated antibody and peroxidase-labeled streptavidin, the staining was developed by reaction with 3. 3′-diamino benzidine for 5 min (ABC kit, Vector Laboratories, Burlingame, CA), and used according to the manufacturer's instructions. The sections were lightly counterstained with hematoxylin. Negative staining of tumor was determined only in the presence of stained uninvolved glands on the same sections. Tumors were considered IRF-1-negative if no signal was detectable in >95% of the tumor.

RESULTS

Members of the TGFβ Family Induce IRF-1 Expression—Affinity-purified recombinant human MIS (35 nM) induced IRF-1 expression in estrogen receptor (ER)-positive T47D and ER-negative MDA-MB-468 breast cancer cell lines (Fig. 1*A*, *upper panels*). Western blot analysis of proteins harvested from T47D cells using an anti-IRF-1 antibody demonstrated the induction of IRF-1 protein by MIS (Fig. 1*A*, *lower left panel*). The specificity of IRF-1 induction by MIS was tested by treating cells with heat inactivated MIS or with the affinity-purified noncleavable, biologically inactive form of rhMIS (L9, 35 nM) that does not induce the regression of the Mullerian duct in organ culture assays (29) or inhibit the growth of T47D cells (4) (Fig. 1*A*, *lower right panel*). MIS-mediated induction of IRF-1 mRNA was also observed in MCF10A cells (Fig. 1*B*, *left panel*), a non-tumorigenic human mammary epithelial cell line with normal karyotype derived from a patient with fibrocystic breast

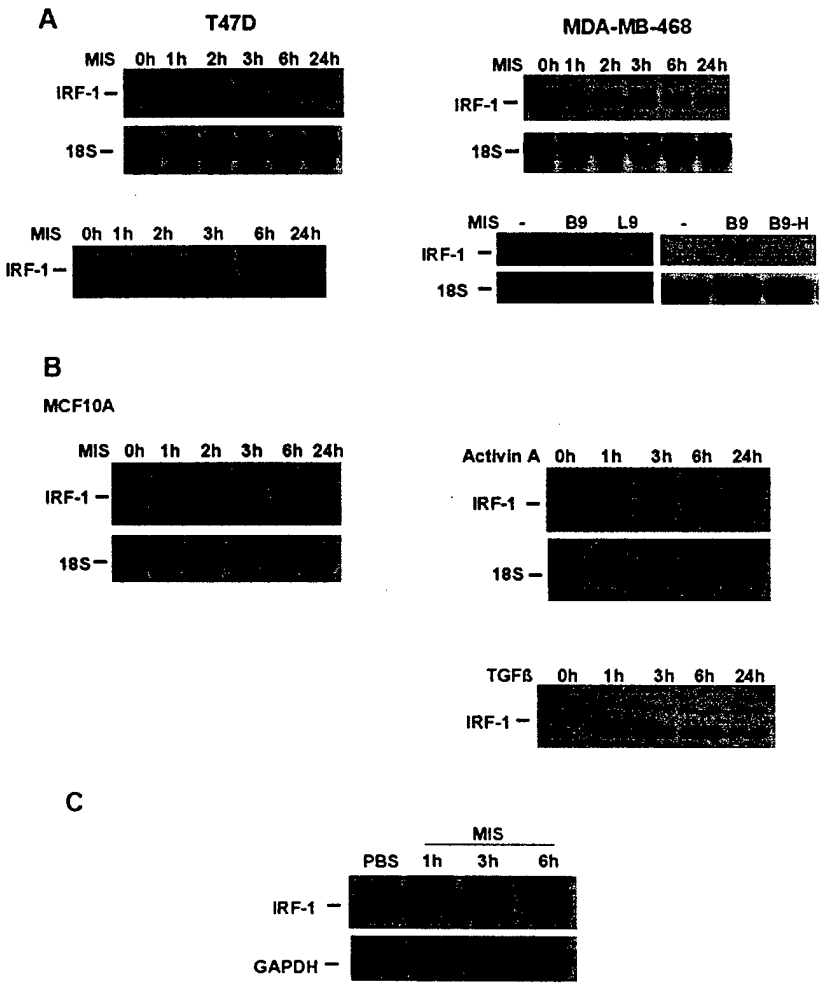


FIG. 1. **Induction of IRF-1 by members of the TGFβ superfamily.** *A*, MIS induces IRF-1 in estrogen receptor-positive and -negative breast cancer cell lines. *Upper panels*, T47D and MDA-MB-468 cells were treated with 35 nM rhMIS for indicated periods of time, and 7.5 μg of total RNA was analyzed by Northern blot using a human IRF-1 probe. *Lower left panel*, total cellular protein lysates (100 μg) harvested from T47D cells treated with 35 nM MIS were analyzed by Western blot using a rabbit anti-IRF-1 antibody. *Lower right panel*, biologically inactive, noncleavable MIS does not induce IRF-1 expression. T47D cells were treated with either 35 nM bioactive MIS (B9) or 35 nM noncleavable biologically inactive rhMIS (L9) or heat-inactivated MIS (B9-H) for 2 h, and total RNA was analyzed for IRF-1 expression. Hybridization to 18 S rRNA is shown to control for loading. *B*, *left panel*, MIS induces IRF-1 expression in MCF10A cells. MCF10A cells were treated with 35 nM MIS and total RNA was analyzed by Northern blot. *Upper right panel*, activin A induces IRF-1 expression in MCF10A cells. MCF10A cells were treated with 2 nM activin A, and total RNA was analyzed by Northern blot. Hybridization to 18 S rRNA is shown to control for loading. *Lower right panel*, TGFβ induces IRF-1 expression in MCF10A cells. MCF10A cells were treated with 100 pM TGFβ, and 50 μg of total protein were analyzed by Western blot using an anti-IRF-1 antibody. *C*, MIS induces IRF-1 mRNA in the mammary glands of mice. Mammary glands of 8-week-old female mice were harvested 1, 3, and 6 h after intraperitoneal injections of 100 μg of MIS/animal, and total RNA was analyzed for IRF-1 expression. RNA isolated from mammary glands of mice 6 h after intraperitoneal injection of PBS was used as control (n = 3 animals for each data point). Hybridization to *GAPDH* is shown to control for loading.

disease (30). Furthermore, both activin and TGFβ induced IRF-1 in MCF10A cells (Fig. 1*B*, *right panels*). Thus IRF-1 expression in mammary epithelial cells may be under the regulation of multiple members of the TGFβ family including MIS.

We next determined whether exposure of mammary glands to exogenous rhMIS would result in the induction of IRF-1 *in vivo*. Intraperitoneal injection of rhMIS into mice induced IRF-1 expression in the mammary glands compared with PBS-injected controls (Fig. 1*C*). The serum rhMIS levels averaged 2–4 μg/ml in the animals as measured by ELISA (26).

Expression of IRF-1 in the Rat Mammary Gland and in Human Breast Cancer—Expression analysis of IRF1 in the mammary glands of virgin, pregnant, lactating, and weaned rats demonstrated that IRF-1 mRNA was detectable in the virgin animals but gradually declined during pregnancy (G5-G21) and reached a nadir at late pregnancy (G17-G21) and lactation (PD0-PD10: lactating). Expression rebounded in the mammary glands of weaned rats (PD3-PD10: weaned) and reached the level observed in virgin animals 3 days after removal of pups (Fig. 2*A*, *upper* and *lower panels*).

Immunostaining with an anti-IRF-1 antibody demonstrated that IRF-1 was expressed predominantly in the epithelial cells of the ducts and lobules of the mammary gland. Expression pattern of the IRF-1 protein coincided with that of the IRF-1 mRNA; expression was detectable in the mammary glands of virgin and early pregnant (G5) animals but not during late pregnancy (G21) or in the lactating glands (Fig. 2*B*, *upper* and *lower panels*). No signal was detected when sections were stained with either affinity-purified rabbit immunoglobulins or with anti-IRF-1 antibody preincubated with the cognate peptide (data not shown).

Since IRF-1 expression in the mammary epithelial cells decreased during pregnancy, a time at which the cells undergo massive proliferation, we wished to determine whether expression of the IRF-1 protein would be lower in breast tumor tissue compared with matched normal glands. We immunostained 23 tumors of various histologic grades with an anti-IRF-1 antibody. Expression of IRF-1 was absent in three tumors but present in the adjacent normal uninvolved ducts and lobules (Fig. 2*C*, *upper panel*). Two of these were of poorly differentiated histologic grade, and one tumor was moderately differentiated. In seven patients, staining for IRF-1 was patchy and limited to 20–80% of the tumor tissue (data not shown). In the other 13 patients expression of IRF-1 was detectable in both the tumor and the surrounding normal tissue (Fig. 2*C*, *lower panel*).

Since these results suggest a growth suppressive effect for IRF-1 in breast cancer, we analyzed whether IRF-1 could induce apoptosis of breast cancer cells. MDA-MB-468 cells in logarithmic growth phase were transiently transfected with a construct, which encodes for the IRF-1 protein, and a plasmid encoding the cell surface marker CD20 as described by Ref. 28. Vector-transfected cells were used as controls. Cells were fixed 72 h after transfection and stained for CD20 expression. An-

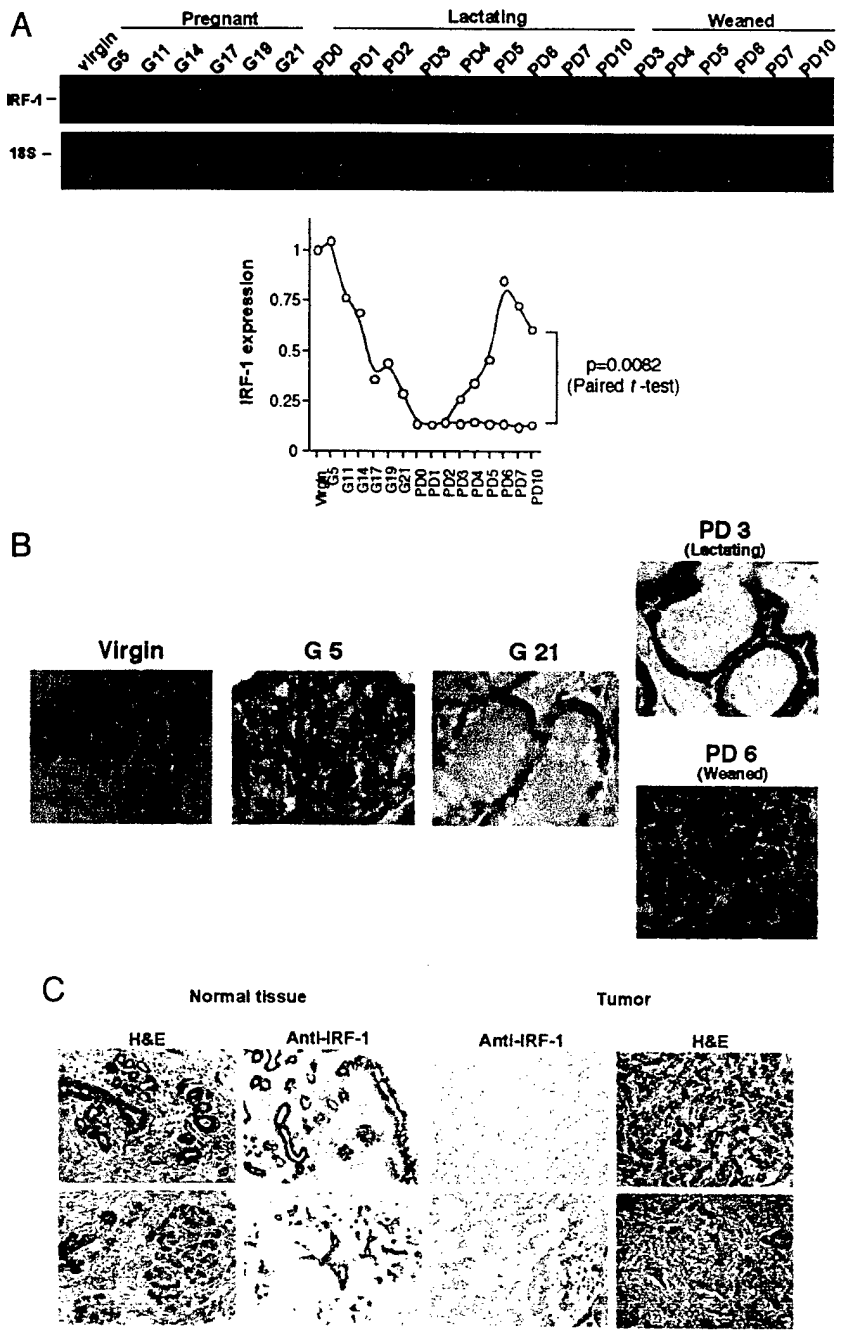


FIG. 2. **IRF-1 expression in rat mammary glands and in human breast cancer.** *A*, IRF-1 expression in the rat mammary gland during postnatal morphogenesis. *Upper panel*, total RNA (7.5 μg) isolated from mammary glands of 8-week-old virgin, pregnant (*G*, Gestation; *G5-G21*) lactating (*PD*, postdelivery; *PD0-PD10*: lactating), and weaned (pups removed 2 days after lactation; *PD3-PD10*: weaned) rats ($n = 1$ for each sample) was analyzed by Northern blot. To measure changes in IRF-1 expression, band intensities were quantified using phosphorimager and iQMac data analysis software. $p < 0.01$ between lactating and weaned groups by Student's paired *t* test. *B*, immunohistochemical detection of IRF-1 protein in the rat mammary gland. Paraffin-embedded mammary tissue sections from various stages of perinatal morphogenesis shown above were stained with an anti-IRF-1 antibody (original magnification ×125). *C*, IRF-1 protein expression in human breast cancer. Paraffin-embedded human breast tumor specimens were immunostained with an anti-IRF-1 antibody. *Upper panel*, IRF-1 is expressed in the adjacent normal ducts and lobules but is undetectable in the tumor (original magnification ×200). *Lower panel*, expression of IRF-1 in both the tumor and matched normal tissue (original magnification ×125). *H&E* stains of tissue sections are included. *D*, IRF-1 induces apoptosis in breast cancer cells. MDA-MB-468 cells were transfected with either vector or an IRF-1 expression construct and a plasmid encoding the cell surface marker CD20. After 72 h, cells were harvested, and Annexin V and DAPI staining of CD20-positive cells were analyzed by FACS. *Upper panels*, representative experiments demonstrating DAPI ± and annexin V ± cells are shown. Zones A (DAPI-negative, annexinV-negative) and B (DAPI-negative, annexin V-positive) represent live and early apoptotic cells, respectively. Zone C represents cells in late stage apoptosis (DAPI-positive, annexin V-positive). *Lower panel* shows the percentage of cells in early (*Zone B*) and early + late stages (*Zones B+C*) of apoptosis ($n = 3$). Statistical analysis was done using Student's *t* test.

nexin V staining of CD20-positive cells demonstrated that expression of IRF-1 in breast cancer cells led to a ~3.5-fold and 5-fold increase in cells in early and late stages of apoptosis, respectively, compared with vector-transfected cells (Fig. 2*D*).

MIS and Interferon Co-stimulate IRF-1—Since IRF-1 is strongly induced by interferons (15) and MIS, we tested the effect of IFN-γ on MIS-mediated induction of IRF-1 expression. IFN-γ induced IRF-1 expression in T47D cells and simultaneous addition of MIS and IFN-γ further increased the induction of the IRF-1 mRNA in both T47D and MDA-MB-468 cells (Fig. 3*A*). We had previously demonstrated that MIS induces the DNA binding activity of NFκB protein complexes in human mammary epithelial cells, breast cancer cells and in the normal breast *in vivo* (3, 4). In order to determine the molecular mechanism by which MIS induces IRF-1 expression in breast cancer cells, gel shift assays were performed using NFκB or STAT-inducing element (SIE) oligonucleotides containing the relevant DNA binding consensus sequences (Fig. 3*B*). As reported previously (3, 4), MIS induced NFκB DNA binding activity consisting of p50 and p65 NFκB subunits in T47D cells. Binding to the SIE DNA sequence was not observed suggesting that MIS does not evoke STAT DNA binding in these cells. IFN-γ however induced SIE DNA binding activity but did not activate the DNA binding activity of NFκB. Antibody supershift experiments demonstrated that the STAT-DNA protein complex induced by IFN-γ contained the STAT-1 protein but not STAT-3 or STAT-5α. In agreement with these results, Western blot analysis of nuclear and cytoplasmic extracts of MIS and IFN-γ-treated T47D cells demonstrated that unlike IFN-γ, MIS did not induce phosphorylation of the STAT1 protein (Fig. 3*C*).

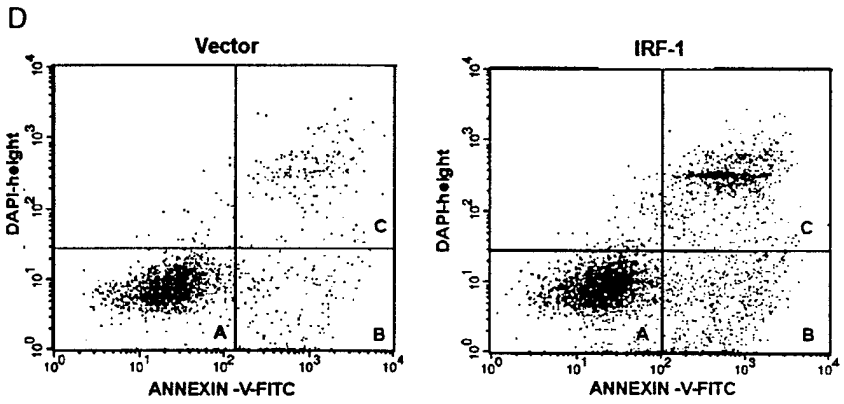
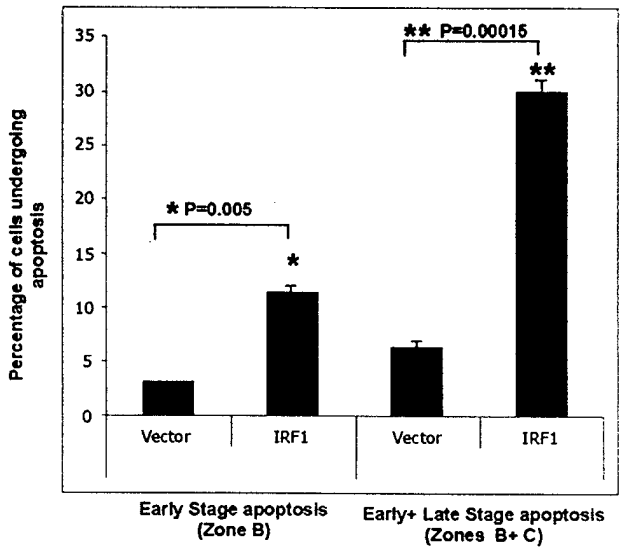


FIG. 2—*continued*



In order to determine whether activation of the NFκB signaling cascade by MIS was responsible for the induction of IRF-1 mRNA, we generated T47D cell clones which express the dominant negative inhibitor of IκB (IκBα-DN). In the rat IκBα-DN transgene used in these experiments, two serine residues at positions 32 and 36 are replaced by alanines. Hence the resulting IκBα-DN protein cannot be phosphorylated in response to activation signals. Thus it functions as a super repressor of NFκB activation (31). Two T47D cell clones expressing the IκBα-DN transgene were identified by the lack of NFκB activation following MIS treatment (Fig. 3*D*, *upper panel*). Induction of IRF-1 by MIS was greatly reduced in the two clones harboring IκBα-DN compared with cells transfected with the empty vector (Fig. 3*D*, *lower panel*). Thus MIS-induced IRF-1 requires activation of NFκB DNA binding activity. We next tested whether co-stimulation of IRF-1 by MIS and IFN-γ would be impaired in cells expressing IκBα-DN. Northern blot analysis of IκBα-DN-expressing cells treated with MIS, IFN-γ or both demonstrated that expression of IκBα-DN did not interfere with IFN-γ-induced IRF-1 expression. The stimulation of IRF-1 mRNA by a combination of MIS and IFN-γ was equivalent to that induced by IFN-γ alone since IRF-1 induction by MIS was impaired in these cells (Fig. 3*E*). Thus it is likely that co-stimulation of IRF-1 by MIS and IFN-γ in breast cancer cells is mediated through activation of NFκB and STAT pathways, respectively. Co-stimulation of IRF-1 was also observed when breast cancer cells were treated with a combination of MIS and IFN-β, a class I interferon (Fig. 3*F*).

Synergistic Induction of CEACAM1 by MIS and IFN-γ Is Mediated by IRF-1—CEACAM1 also known as biliary glycoprotein (BGP) is a Ca^{2+}-dependent cellular adhesion molecule that is expressed in epithelial cells (32, 33). An interferon-sensitive response element (ISRE) in the *CEACAM1* promoter is specifically protected by IRF-1 in DNA footprints and is required for induction of a *CEACAM1* promoter-driven reporter construct by IRF-1 (34). Both MIS and IFN-γ induced CEACAM1 expression in T47D cells (Fig. 4*A*, *left panel*). Induction of CEACAM1 by these two ligands was also observed in MDA-MD-468 cells (data not shown). Interestingly, simultaneous addition of MIS and IFN-γ resulted in synergistic induction of CEACAM1 expression (Fig. 4*A*, *right panel*).

In order to determine whether induction of CEACAM1 by MIS and IFN-γ was mediated through IRF-1, we generated T47D cells that stably express the antisense IRF-1 transcript. The ability of antisense IRF-1 to block the translation of IRF-1 protein was demonstrated by transient transfection of sense and antisense IRF-1 constructs into COS cells (Fig. 4*B*). Antisense IRF-1 expression inhibited the translation of IRF-1 protein derived from an IRF-1 expression construct. Phosphorimager analysis of CEACAM1 mRNA induction in breast cancer cells expressing antisense IRF-1 (Fig. 4*C*, *upper panel*) demonstrated that MIS- and IFN-γ-induced CEACAM1 expression by

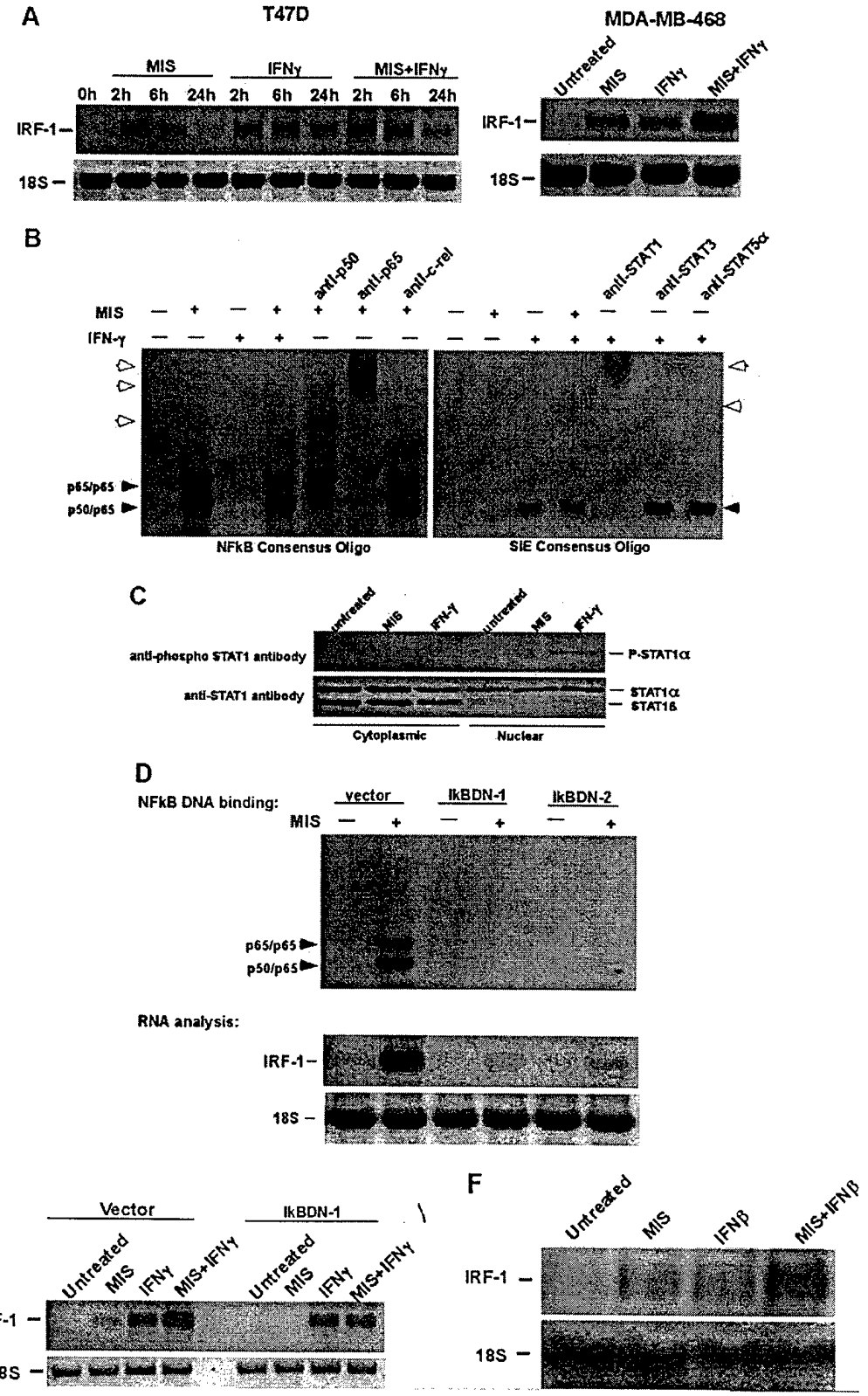


FIG. 3. **MIS and interferon co-stimulate IRF-1 expression.** *A*, T47D cells were treated with 1 ng/ml of IFN-γ or 35 nM MIS or a combination of 35 nM MIS and 1 ng/ml of IFN-γ for increasing periods of time. Total RNA isolated from cells was analyzed by Northern blot. *Right panel*, MDA-MB-468 cells were treated with 0.2 ng/ml of IFN-γ or 17.5 nM MIS or a combination of MIS and IFN-γ for 6 h and IRF-1 expression was analyzed by Northern blot. Hybridization to 18 S rRNA is shown. *B*, T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ or both for 1 h, and 3 μg of nuclear proteins were analyzed by gel-shift assay using ^{32}P-labeled oligonucleotides containing the consensus DNA binding site for NFκB or the STAT proteins. Positions of the DNA protein complexes (*closed arrows*) and the antibody-supershifted complexes (*open arrows*) are indicated. *C*, T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ for 1 h, and 30 μg of nuclear and cytoplasmic proteins were analyzed

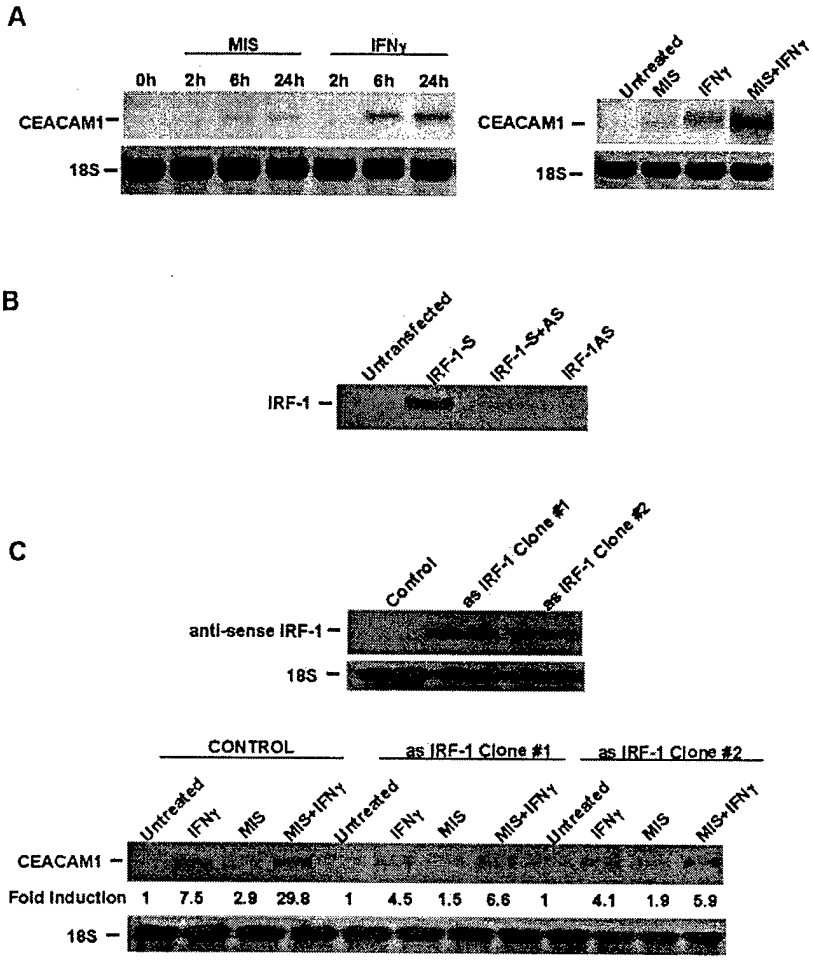


FIG. 4. **Synergistic induction of CEACAM1 by MIS and IFN-γ is mediated by IRF-1.** *A, left panel,* T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ for increasing periods of time. Total RNA isolated from cells was analyzed for CEACAM1 expression by Northern blot. Hybridization to 18 S rRNA is shown. *Right panel,* T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ or both for 24 h. Total RNA isolated from cells was analyzed for CEACAM1 expression. Hybridization to 18 S rRNA is shown. *B,* antisense IRF-1 ablates translation of the IRF-1 protein. Lysates from COS cells transiently transfected with 1 μg of CMV-driven sense (*IRF-1-S*) or 2.9 μg of antisense IRF-1 (*IRF-1AS*) constructs or 2.9 μg of antisense + 1.0 μg of sense IRF-1 constructs were immunoblotted with an antibody to IRF-1. Position of the IRF-1 protein is indicated. *C,* synergistic induction of CEACAM1 by MIS and IFN-γ is impaired in T47D cells expressing antisense IRF-1. *Upper panel,* Northern blot analysis of total RNA isolated from T47D cells stably transfected with antisense IRF-1 demonstrates the expression of antisense IRF-1 transcript. *Lower panel,* cells were induced with 35 nM MIS or 1 ng/ml of IFN-γ or both for 24 h. Total RNA was analyzed by Northern blot for CEACAM1 expression. Fold change in CEACAM1 expression quantified using phosphorimager and iQMac data analysis software is shown below.

3- and 8-fold, respectively, in control cells and by 2- and 5-fold, respectively, in two clones expressing the antisense IRF-1 transcript suggesting that antisense IRF-1 slightly lowered CEACAM1 induction by MIS or IFN-γ (Fig. 4*C, lower panels*). However, the synergistic up-regulation of CECAM1 mRNA by combined treatment with MIS and IFN-γ was greatly impaired by the expression of antisense IRF-1; MIS and IFN-γ together induced CEACAM1 expression by 30-fold in control cells while its induction was additive (6–7-fold) in both clones expressing antisense-IRF-1 RNA (Fig. 4*C, lower panel*).

Effect of MIS and IFN-γ on Breast Cancer Cell Growth—Since the signaling events initiated by MIS and IFN-γ converge to increase the magnitude of gene expression, we next tested their effect on the growth of breast cancer cells. Treatment of MDA-MB-468 cells with either MIS or IFN-γ inhibited growth and the presence of both inhibited growth better (Fig. 5*A*; $n = 8$).

In order to identify the mechanism by which MIS and IFN-γ inhibit growth, assays to estimate cell cycle progression and apoptosis were performed. MDA-MB-468 cells were treated with MIS, IFN-γ, or MIS+IFN-γ for 72 h, and the fraction of cells in each phase of the cell cycle was estimated by fluorescence-activated cell sorting (Fig. 5*B*). Compared with untreated cells, MIS or IFN-γ treatment consistently led to a statistically significant decrease in the number of cells in the S-phase of the cell cycle ($p < 0.001$ by Student's t test). Interestingly, in cultures treated with a combination of MIS and IFN-γ, the percentage of cells in the S-phase did not demonstrate a greater decrease compared with that seen with either agent alone and these cultures did not exhibit any other extensive alteration in cell cycle distribution compared with cells treated with either agent alone. Thus the enhanced inhibition of breast cancer cell growth by MIS and IFN-γ could not be explained by combined changes in cell cycle progression compared with treatment with either agent alone.

Translocation of annexin V from the inner surface of the plasma membrane to the outside occurs after initiation of apoptosis and thus serves as a marker of apoptosis. MDA-MB-468 cells were treated with MIS, IFN-γ, or MIS+IFN-γ for 96 h and cell surface expression of annexin V was analyzed by staining with a FITC-annexin V antibody. Quantification of annexin V-positive cells demonstrated that IFN-γ is a strong inducer of

by Western blot. *Upper panel,* Immunoblot analysis with an antiphospho-STAT1 antibody. *Lower panel,* the blot was stripped and reanalyzed with an anti-STAT1 antibody. Positions of STAT1α and STAT1β are indicated. IFN-γ specifically induced the phosphorylation of STAT1α. *D,* MIS induces IRF-1 through activation of NFκB. T47D cells stably transfected with either vector or IκBα-DN were treated with MIS for 0 and 2 h. *Upper panel,* nuclear proteins were analyzed by gel-shift assay to determine NFκB DNA binding activity. Positions of the NFκB, DNA protein complexes are indicated. *Lower panel,* total cellular RNA (7.5 μg) was analyzed for induction of IRF-1. Hybridization to 18 S rRNA is shown as control for loading. *E,* vector and IκBα-DN-expressing T47D cells were treated with 35 nM MIS or 1 ng/ml of IFN-γ or both for 2 h, and total RNA (5 μg) was analyzed for IRF-1 expression. *F,* MIS and IFN-β co-stimulate IRF-1 expression. T47D cells were treated with 1 ng/ml of IFN-β or 17.5 nM MIS or a combination of 17.5 nM MIS and 1 ng/ml of IFN-β for 2 h. Total RNA isolated from cells was analyzed by Northern blot.

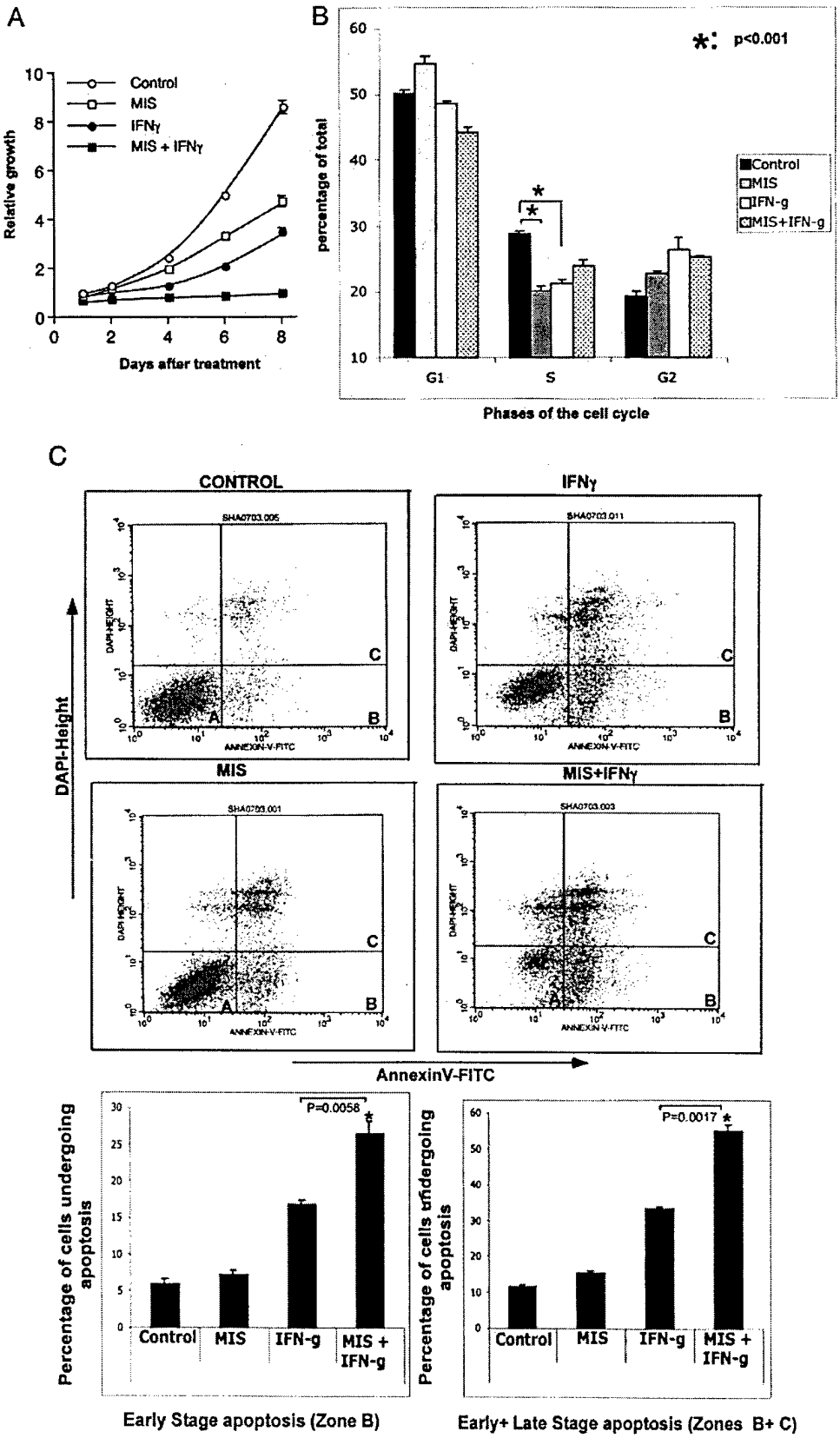


FIG. 5. **MIS promotes IFN-γ-induced apoptosis in breast cancer cells.** *A*, MIS and IFN-γ were added at a concentration of 35 nM and 5 ng/ml, respectively, to MDA-MB-468 cells seeded in a 96-well plate. Cell viability was determined after 1, 2, 4, 6, and 8 days by analysis of MTT conversion. Plates were analyzed in an ELISA plate reader at 550 nm with a reference wavelength of 630 nm ($n = 8$). *B*, cell cycle analysis of MDA-MB-468 cells treated with MIS and IFN-γ. Cells were treated with 35 nM MIS and 5 ng/ml IFN-γ or both for 72 h, fixed in ethanol, and

apoptosis in breast cancer cells (Fig. 5*C*). MIS consistently increased apoptosis in several experiments but its effect was much less potent than that of IFN-γ at the concentration tested. However, treatment of cells with a combination of MIS+IFN-γ together resulted in a synergistic increase in the fraction of cells in early and late stages of apoptosis. Thus growth inhibition of MDA-MB-468 cells following co-treatment with MIS and IFN-γ results from enhanced of apoptosis rather than a combinatorial effect on the cell cycle.

DISCUSSION

MIS is a sexually dimorphic hormone that plays an important role in proper sexual development in male embryos (1). Interferons are antiviral and immunoregulatory proteins, which can negatively regulate growth in various cell types (35). IRF-1 mediates many IFN-γ-induced responses within cells by enhancing gene expression (14, 15) and its expression is also modulated by the cytokines TNF-α, IL-1, IL-6, and prolactin (15). TGFβ can either up- or down-regulate the expression of IRF-1 depending on its growth regulatory role in a particular cell type. In human embryonic lung fibroblasts, TGFβ-stimulated DNA synthesis was associated with suppression of IRF-1 expression whereas in human cholangiocarcinoma cells, TGFβ suppressed DNA synthesis through up-regulation of IRF-1 (36). Our results demonstrate that in addition to TGFβ, MIS and activin A also induce IRF-1 suggesting that members of the TGFβ superfamily may represent another class of molecules that can regulate IRF-1 expression.

Analysis of IRF-1 expression in the rat mammary gland demonstrated a gradual decline in mRNA that begins at the early stages of pregnancy suggesting that it may be a negative regulator of growth and/or differentiation in mammary epithelial cells. The RNA and protein were almost undetectable during late stages of pregnancy and lactation but recovered to levels seen in virgin animals nearly 3 days after removal of pups. However, Chapman *et al.* (37) analyzing total protein isolated from mammary glands of lactating and weaned mice by Western blot demonstrated that IRF-1 protein was expressed in the lactating mammary glands of mice and that levels did not change significantly during 24, 48, 72, and 96 h of involution. Western blot analysis is a more sensitive analytical tool than immunostaining to detect low levels protein expression. Thus it is possible that the discrepancy between these two observations results from the difference in sensitivity between the two techniques. Alternatively, the difference could also be attributed to the samples analyzed; total IRF-1 expression in the mammary gland (37) *versus* expression in the epithelial compartment in which IRF-1 protein expression is maintained at very low levels during late pregnancy, lactation, and early stages of involution but is up-regulated at weaning.

Many lines of evidence demonstrate that IRF1 plays a key role in growth control (25). The *IRF-1* gene maps to the chromosomal region 5q31.1 that is frequently deleted in human leukemia (38). The tumor suppressor activity of IRF-1 is also suggested by loss of an *IRF-1* allele in esophageal and gastric cancer (39–41). IRF-1 immunostaining of breast cancer specimens demonstrated that the protein was not detectable in 14% of invasive tumors. Expression did not correlate with estrogen receptor status or Page grade but correlated with nuclear grade; it was undetectable in 41% of breast tumors of high nuclear grade (42). Consistent with the results reported by Doherty *et al.* (42), our results demonstrated loss of IRF-1 expression in 13% (3/23) of breast tumors compared with matched normal control tissue. Furthermore, 7 tumors demonstrated patchy staining in 20–80% of the tumor tissue. Thus some breast cancers may by-pass the growth-inhibitory effect exerted by IRF-1 by down-regulating its expression. In agreement with this concept, expression of IRF-1 in breast cancer cells results in the robust induction of apoptosis.

Paradoxically, examination of involuting mammary glands of IRF-1-null mice demonstrated accelerated apoptosis compared with wild-type mice at 48 h of involution. However, no difference in morphology was evident in the mammary glands isolated from control and IRF-1-null mice at 72 h of involution (37). These results suggest that IRF-1 may be a suppressor of premature epithelial apoptosis in the mammary gland. Thus it is possible that IRF-1 serves different functions during various stages of postnatal mammary gland development, neoplastic transformation, and tumorigenic process of the breast.

Induction of IRF-1 by IFN-γ occurs through phosphorylation of the latent transcription factor STAT1, homodimers of which bind to the *IRF-1* promoter (15). However, the presence of a putative NFκB site within the *IRF-1* promoter (43, 44) renders it responsive to extracellular signals that activate the NFκB pathway. Induction of IRF-1 by MIS in breast cancer cells was mediated by activation of NFκB and addition of methylthioadenosine to inhibit STAT1 methylation (45) lowered IFN-γ-induced IRF-1 expression (data not shown). Thus IRF-1 co-stimulation by MIS and IFN-γ in breast cancer cells may occur through activation of these two pathways.

Several growth regulatory genes including those with antiproliferative activity such as IFNα/β, p21, and CEACAM1 have IRF-1 DNA recognition sites in their promoters (25, 34). In HeLa and HT-29 cells, IFN-γ up-regulated a *CEACAM1* promoter-driven-luciferase construct by 2- and 2.5-fold, respectively, an effect that was abrogated upon mutating the interferon response element that binds IRF-1 (34). However, this report did not evaluate the effect of IFN-γ and IFN-γ-induced IRF-1 on the induction of endogenous CEACAM1 mRNA. In breast cancer cells expression of antisense IRF-1, which ablates translation of the IRF-1 protein decreased CEACAM1 induction slightly when MIS and IFN-γ were used alone suggesting that IRF-1 may be partially responsible for this inductive process. Quantification of band intensities demonstrated that induction of CEACAM1 by a combination of MIS and IFN-γ was strictly additive in T47D cells expressing antisense IRF-1. However, the synergistic up-regulation of CEACAM1 by MIS and IFN-γ was completely abrogated in both T47D cell clones stably expressing the antisense IRF-1 transcript suggesting that IRF-1 may be involved in the interaction between MIS and IFN-γ leading to the synergistic induction of CEACAM1.

IRF-1 has been implicated in mediating the IFN-γ contribution to synergistic enhancement of transcription in other experimental systems. Enhancer elements that bind IFN-γ-responsive transcription factors including an IRF-1 binding site have been shown to be involved in the synergistic induction of the *iNOS* promoter-driven luciferase construct by IFN-γ (46).

incubated with propidium iodide and RNase A. DNA content was analyzed by FACS. Cell cycle analysis of untreated cells grown for 72 h is shown as control. Statistical analysis was done using Student's *t* test. *C*, MIS promotes IFN-γ-induced apoptosis. MDA-MB-468 cells were treated with MIS and IFN-γ at a concentration of 35 nM and 5 ng/ml, respectively for 96 h. Cells were stained with annexin V-FITC, and DAPI and analyzed by FACS. *Upper panels*, representative experiments demonstrating DAPI ± and annexin V ± cells are shown. Zones A (DAPI-negative, annexin V-negative) and B (DAPI-negative, annexin V-positive) represent live and early apoptotic cells, respectively. Zone C represents cells in late stage apoptosis (DAPI-positive, annexin V-positive). *Lower panel* shows the percentage of cells in early (*Zone B*) and early + late stages (*Zones B+C*) of apoptosis ($n = 3$). Statistical analysis was done using Student's *t* test.

In IRF-1-null macrophages, the ability of IFN-γ to up-regulate as well as synergistically induce Cox-2 mRNA expression was abrogated (47). The synergistic induction of transcription by IRF-1 has been shown to depend on protein-protein interaction (48–50). Further analysis of the *CEACAM1* promoter-driven reporter construct may be required to delineate the process by which IRF-1 mediates the synergistic interaction between MIS and IFN-γ in the induction of the *CEACAM1* gene in breast cancer cells.

CEACAM1, located on chromosome 19 (51), is down-regulated in human colon and prostate cancers (52, 53) and in about 30% of breast carcinomas (54, 55). Consistent with its tumor suppressor function, introduction of CEACAM1 into MDA-MB-468 cells suppressed tumorigenicity in nude mice (56). In normal mammary epithelial cells, CEACAM1 staining is confined to the luminal surface and its localized expression appears to be important in lumen formation (55, 57) suggesting that CEACAM1 expression may be important in differentiation of mammary epithelial cells. Furthermore, expression of CEACAM1 in the BGP-negative MCF7 cells induces cell death with occasional formation of acini when grown in extracellular matrix (57). The synergistic up-regulation of CEACAM1 by MIS and IFN-γ suggests that the level of CEACAM1 expression in the mammary epithelial cells may depend on the integrated response to various extracellular signals received by the cell. Whether the synergistic induction of CEACAM1 by MIS and IFN-γ can reinitiate the differentiation program in breast cancer cells remains to be determined.

IFN-γ in combination with IFN-β has been shown to induce the regression of human breast cancer cell lines MCF7 and BT20 grown as xenografts in nude mice (58). Although the antitumor effect of IFN-γ *in vivo* has been well documented, toxicity associated with exposure to IFN-γ has diminished its utility in treatment (59). The ability of MIS to augment IFN-γ-induced growth inhibitory/differentiation signals such as CEACAM1 and apoptosis of breast cancer cell growth, suggests that MIS may prove to be beneficial in harnessing the antitumor effects of this cytokine, especially since high levels of MIS have not shown any harmful effects in humans (60).

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