Müllerian Inhibiting Substance Regulates NFκB Signaling and Growth of Mammary Epithelial Cells in Vivo*

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Müllerian inhibiting substance (MIS) inhibits breast cancer cell growth in vitro through interference with cell cycle progression and induction of apoptosis, a process associated with NFκB activation and up-regulation of one of its important target genes, IEX-18 (Segev, D. L., Ha, T., Tran, T. T., Kenneally, M., Harkin, P., Jung, M., MacLaughlin, D. T., Donahoe, P. K., and Maheswaran, S. (2000) J. Biol. Chem. 275, 28371–28379). Here we demonstrate that MIS activates the NFκB signaling cascade, induces IEX-18 mRNA, and inhibits the growth of MCF10A, an immortalized human breast epithelial cell line with characteristics of normal cells. In vivo, an inverse correlation was found to exist between various stages of mammary growth and MIS type II receptor expression. Receptor mRNA significantly diminished during puberty, when the ductal system branches and invades the adipose stroma and during the expansive growth at lactation, but it was up-regulated during involution, a time of regression and apoptosis. Peripartum variations in MIS type II receptor expression correlated with NFκB activation and IEX-18 mRNA expression. Administration of MIS to female mice induced NFκB DNA binding and IEX-18 mRNA expression in the breast. Furthermore, exposure to MIS in vivo increased apoptosis in the mouse mammary ductal epithelium. Thus, MIS may function as an endogenous hormonal regulator of NFκB signaling and growth in the breast.

The importance of MIS, a sexually dimorphic member of the transforming growth factor β family of hormones, in regression of the Müllerian duct in male embryos is well established. MIS is produced by Sertoli cells of the testis even after regression of the Müllerian duct and continues to be made throughout adulthood. In females, synthesis by granulosa cells of the ovary commences after birth and persists until menopause (2, 3). The continued production of MIS throughout adolescence and adulthood in males and females implies other functional roles for this hormone after causing regression of the Müllerian duct.

The MIS type II receptor, a highly conserved single transmembrane serine threonine kinase, is homologous to members of the transforming growth factor β family of type II receptors (4–6). The binding of MIS ligand to its receptor initiates a signaling cascade that is dependent on recruitment of type I receptors, ALK2 and ALK6, which also signal for bone morphogenetic proteins (7–9). The MIS type II receptor gene contains 11 exons and encodes a 1.8-kilobase mRNA. It is expressed at high levels in the Müllerian duct, the Sertoli and granulosa cells of embryonic and adult gonads (6). However, the status of receptor expression and MIS responsiveness in other tissues has yet to be clarified.

Using various different techniques, we recently demonstrated MIS type II receptor expression in normal breast, human breast fibroadenomas, ductal carcinomas, and cancer cell lines (10). MIS inhibited the growth of both estrogen receptor-positive and estrogen receptor-negative human breast cancer cells in vitro by interfering with cell cycle progression and inducing apoptosis. The effect of MIS on breast cell proliferation correlated with its ability to induce the NFκB family of transcription factors and to up-regulate IEX-18 (10), an immediate early gene known to be induced following NFκB activation by other extracellular signals. PRG1 and gly96 represent the rat and mouse homologues, respectively, of human IEX-1 (11). Overexpression of IEX-1 in breast cancer cells inhibited their growth, indicating a negative growth regulatory role for this newly identified NFκB-inducible gene (10).

The NFκB family consists of transcriptional activators, including p65, p50, p52, and c-Rel, that share a Rel homology domain and form either homo- or heterodimers that bind to DNA in a sequence-specific manner. NFκB in its inactive state exists in the cytosol bound to the inhibitory IκB family of molecules. Activation of the pathway by extracellular signals leads to phosphorylation and degradation of IκB with subsequent nuclear localization of NFκB (12, 13). Expression of a dominant negative inhibitor of NFκB (IκBα-DN) in breast cancer cells ablated MIS-mediated induction of IEX-18, inhibition of growth, and induction of apoptosis, indicating that activation of the NFκB pathway was required for these processes (10).

In order to determine whether MIS-mediated growth inhibition through activation of the NFκB signaling cascade, previously characterized using human breast cancer cell lines, is also functional in normal breast tissue, we analyzed the effect...
of MIS on MCF10A, a nontumorigenic breast epithelial cell line (1), and on murine mammary glands in vivo. Furthermore, to evaluate whether these events are developmentally regulated, we analyzed endogenous MIS type II receptor expression, NFκB activity, and IEX-1 expression in the mammary gland during postnatal morphogenesis. In this report, we demonstrate that NFκB activates the NFκB signaling cascade, induces IEX-1 expression, and inhibits the growth of MCF10A cells. Peripartum expression of MIS type II receptor in the rat breast correlated with the level of NFκB DNA binding activity and expression of IEX-1 mRNA. In addition, exogenous MIS activated NFκB DNA binding and induced IEX-1 expression in the mammary glands of adult mice and increased the number of apoptotic cells in the ductal epithelium of the breast in vivo. Thus, MIS might be a hormonal regulator of the NFκB signaling cascade in vivo and a negative regulator of normal breast growth.

EXPERIMENTAL PROCEDURES

Cell Lines and Growth Inhibition Assays—Human breast cancer cell line T47D was grown in Dulbecco's modified medium supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. MCF10A cells were grown in mammary epithelial growth medium (Clonetics) supplemented with 100 ng/ml cholera toxin (Calbiochem). 184A1 cells (a gift from Dr. Martha Stampfer) were grown in mammary epithelial growth medium supplemented with isoproterenol and transferrin.

To test the growth inhibitory effect of exogenous MIS, MCF10A cells were seeded in 100-mm tissue culture flasks in the absence of MIS. MIS was added after 24 h, cultures were grown in the presence or absence of 35 nM MIS for 3 days, and cell numbers were compared by Coulter counter.

Animals, MIS, and MIS Treatment—MIS type II receptor expression analyses in the rat breast during development and peripartum stages were done using Harlan Sprague-Dawley rats. Recombinant human MIS (rhMIS) was collected from growth medium of Chinese hamster ovary cells transfected with the human MIS gene and purified as described in Ref. 14.

To study the effects of rhMIS on the mammary gland, adult female C3H mice (8 weeks old; average weight, 25 g) were obtained from the Edwin L. Steele Laboratory, Massachusetts General Hospital (Boston, MA). All animals were cared for and experiments performed in accordance with protocols approved by the Committee on the Use of Laboratory Animals of the Massachusetts General Hospital. All experiments were performed using ketamine/xylazine (100/10 mg/kg) for anesthesia. Each animal was placed in a metabolic cage for 1 h. Cells were harvested in cold PBS, resuspended in 1 ml of TKM (3.1(–) plasmid. The resulting construct was sequenced to confirm the integrity of yeast tRNA was incubated without RNase to control for probe degradation.

To determine whether MIS could also inhibit the growth of non-tumorigenic breast epithelial cells, we analyzed MCF10A and T47D cells with the Peripartum expression of MIS type II receptor in the rat breast and at various stages of development using the RPA III ribonuclease protection assay kit (Ambion). RNase protection assays were done with 90–100 μg of total RNA isolated from the rat breast at various stages of development using the RPA III ribonuclease protection assay kit (Ambion). RNA samples derived from both individual animals or pooled from groups of three animals were analyzed as indicated in the figure legends. Briefly, RNA was hybridized with 75–80 ng of radiolabeled probe overnight at 50–55 °C and digested with a mixture of RNase A and RNase T1 for 30 min at 37 °C. The protected fragments were precipitated and analyzed on a denaturing 6% polyacrylamide/6 M urea gel. The same amount of yeast tRNA was used as a positive control for the function of RNase, and another sample containing the same amount of yeast tRNA was incubated without RNase to control for probe degradation.

The riboprobe to detect the long and short forms of PRG1/IEX-1 mRNA in the rat was generated by PCR amplification using the following primers: sense, 5′-CAC GGAT CTT CGG ACA AGA GAA C TT CCT T-3′; antisense, 5′-CCT CCT GAG CCT GCT GAG CAT ATC TAC CCC-3′. cDNA generated from RNA isolated from the mouse testis was used as the template. RNase protection was done as described above.

To determine whether the long and short forms of mouse gly96/IEX-1 mRNA were detectable in the rat brain, a PCR-based assay was used. PCR amplification was done with the following primers: sense, 5′-ACC CTC CAC ACC ATG ATG ACT G-3′; antisense, 5′-CCT CCT TCA GCC ATC AAA ATC TGG-3′. Rat genomic DNA was used as the template. The resulting fragment was cloned into SmI sites of the pCR-Script Amp sk (+) plasmid. The construct was linearized with SalI, and the antisense transcript was obtained with T3 polymerase (MAXScript in vitro transcription kit, Ambion). RNase protection was done as described above.

Primers for detecting MIS type II receptor expression in MCF10A and 184A1 cells were as follows: sense, 5′-GCC CGA ATT CGT CCC AGA GAA CAT TCT T-3′; antisense, 5′-CGG CCT CGA GTC TCT GAG CAT GAT TAC CCC-3′. cDNA generated from RNA isolated from the mouse testis was used as the template. RNase protection was done as described above.

Northern Blot and PCR Analyses—RNA was isolated from cells or tissue samples using RNA STAT-60 total RNA isolation kit (Tel-Test, Inc.). Indicated amounts of RNA were separated on a formaldehyde gel, transferred to HyBond membrane (Amersham PharmaBiotech), and probed with either PCR-amplified human MIS type II receptor reverse primers or PCR-amplified mouse MIS type II receptor primers. The Northern blot was probed with human IEX-1 or mouse gly96/IEX-1 as indicated in the figure legends. The mouse gly96/IEX-1 probe for Northern analysis was derived by PCR amplification as previously described (10).

Apoptosis Assays—Breast tissue was harvested, fixed, and embedded in paraffin. After sectioning and deparaffinization, apoptotic cells were detected using a fluorescein in situ cell death detection. Nuclear DNA was used as the template. The resulting fragment was cloned into SmI sites of the pCR-Script Amp sk (+) plasmid. The construct was linearized with SalI, and the antisense transcript was obtained with T3 polymerase (MAXScript in vitro transcription kit, Ambion). RNase protection was done as described above.

To determine whether MIS could also inhibit the growth of non-tumorigenic breast epithelial cells, we analyzed MCF10A cells,
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with exogenous MIS inhibited growth by 60% (Fig. 1A). Reverse transcription-PCR analysis demonstrated the presence of receptor in both MCF10A and 184A1 cells (Fig. 1A). Sequence analysis of the 582-base pair DNA fragment was identical to exons 1, 2, 3, 4, and 5 of the human MIS type II receptor (data not shown).

Treatment of MCA10A cells with MIS induced three NFκB DNA-protein complexes following 1 h of treatment (Fig. 1B, left panel). The heavier complex comigrated with the NFκB DNA-protein complex stimulated in T47D cells following MIS treatment (Fig. 1B, right panel). Simultaneous addition of either rabbit anti-p50 or anti-p65 antibodies to the binding reaction demonstrated that the heaviest complex consisted predominantly of p50 and p65 subunits, as was demonstrated in T47D cells (10). The faster migrating complexes, however, were unique to MCF10A cells and contained p50 subunits. Incubation of nuclear lysates with anti-c-Rel antibody did not supershift the complexes, suggesting that c-Rel protein was not present in them.

We next investigated whether MIS-mediated increase in NFκB DNA binding activity in MCF10A cells correlated with the induction of its target gene IEX-1S. An estimated 5-fold induction of IEX-1 mRNA was observed following 1 h of treatment with MIS, suggesting that the NFκB binding activity induced by MIS was functionally active (Fig. 1C, top panel). Reverse transcription-PCR analysis of the MIS-treated samples demonstrated that MIS selectively up-regulated the IEX-1S transcript (Fig. 1C, bottom panel). We had previously demonstrated that MIS inhibited the growth of breast cancer cells in vitro through a NFκB-mediated mechanism. As with breast cancer cell lines, treatment of MCF10A cells in vitro with exogenous MIS inhibited growth by 60% (Fig. 1D).

Regulation of MIS-mediated Signaling during Mammary Gland Development in Vivo—To confirm that MIS-mediated signaling events and its effects on breast epithelial cell growth identified using the in vitro cell systems are functional in vivo, we investigated whether MIS type II receptor is expressed in the normal breast. Total RNA isolated from the mammary glands of 8-week-old mice was analyzed by RNase protection assay using an antisense riboprobe specific for exon 11 and 3’ untranslated region of the mouse MIS type II receptor (Fig. 2A, left panel). The protected fragment was 89 base pairs shorter than the probe due to unrelated sequences at the 5’ and 3’ ends. Detection of a protected fragment of the expected size in the breast, which comigrated with that from the testis, confirmed that the MIS type II receptor mRNA was expressed in normal breast but at a level much lower than that in the testis (Fig. 2A, right panel).

MIS type II receptor was also detected in rat breast by RNase protection assay using an antisense riboprobe that contained exon 11 and the 3’ untranslated region specific to the rat MIS type II receptor DNA sequence. Because mammary tissue undergoes the majority of its development in the adult and robust expansion of the breast epithelium occurs during pregnancy and continues into lactation, MIS type II receptor mRNA levels were analyzed using total RNA isolated from mammary glands of virgin, pregnant, lactating, and weaned rats (Fig. 2B). Phosphorimaging of band intensities from three independent experiments demonstrated an 80% decrease in MIS type II receptor expression 2 days after delivery during early lactation. The receptor mRNA rebounded to higher levels 2 days after removal of pups, a period of ductal regression (Fig. 2B, bottom panel).

Analysis of MIS type II receptor expression during breast development in Harlan Sprague-Dawley rats revealed a grad-
FIG. 2. MIS type II receptor expression analyses in the mammary gland. A, left panel, schematic representation of the mouse MIS type II receptor antisense riboprobe used for RNase protection assay. Right panel, MIS type II receptor expression in normal mouse breast. Total RNA (90 μg) isolated from 8-week-old female mouse mammary glands was analyzed by RNase protection assay. The protected fragments after digestion with RNase were analyzed on a 6% denaturing polyacrylamide gel. 90 μg of yeast tRNA was hybridized with the probe and incubated with or without RNase to test the activity of RNases and probe integrity, respectively. A small aliquot of the probe was loaded directly on the gel to control for integrity of the probe. Equal amount of total RNA from mouse testis was analyzed as a positive control. Positions of the 341-nucleotide (nt) probe and the protected fragment (252 nt) are indicated. B, regulation of MIS type II receptor expression during postnatal morphogenesis of the rat.
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NFκB binding activity in the breast increases slightly during pregnancy, with undetectable levels during lactation (21, 22), and is robustly induced in the involuting mammary gland, with the highest levels of binding evident at 2–3 days of involution (21). In order to determine whether activation of NFκB DNA binding correlated with changes in MIS type II receptor expression during breast development in rats, electromobility gel shift assays were performed. NFκB DNA binding activity was quite readily detectable in the mammary glands of both virgin and pregnant rats. Consistent with the results in mice, reported by Clarkson et al. (21), very little NFκB activation was observed in the mammary glands of rats during early lactation, when type II receptor expression was lowest, but was strongly induced 2 days after removal of pups, a period of ductal involution, when type II receptor expression rebounds, indicating a correlation of NFκB activation with peripartum MIS type II receptor levels (Fig. 2D).

Because MIS-induced NFκB activation in human breast cancer cell lines led to specific up-regulation of its target gene IEX-1S, we tested whether activation of NFκB during different phases of mammary morphogenesis in the rat correlated with the levels of IEX-1 mRNA. As demonstrated in Fig. 2E, top panels, PRG1/IEX-1S mRNA expression diminished in the lactating breast but returned to levels observed in the breasts of virgin animals after 2 days of postlactational involution, paralleling NFκB activation during this period of breast morphogenesis. Ethidium bromide staining of the gel demonstrated equal loading of RNA. PCR primers that permit the differentiation of the long and short PRG1 transcripts predominantly detected the latter in the normal breast, with a minor band of 403 base pairs (Fig. 2E, middle panel). PRG1/IEX-1L has an in-frame insertion of 37 amino acids resulting from the presence of the entire intronic sequence within the coding region of the PRG1/IEX-1S transcript (23). To rule out the possibility that the 403-base pair band could have resulted from contaminating genomic DNA being amplified by PCR, a nonquantitative DNA amplification technique, RNase protection assay using an antisense riboprobe specific for rat PRG1/IEX-1L (Fig. 2E, Scheme), was performed. The results confirmed that PRG1/IEX-1S was the predominant transcript expressed throughout development (Fig. 2E, bottom panel). A protected band of 403 nucleotides that would correspond to PRG1/IEX-1L was not detected. The developmental regulation of PRG1/IEX-1S coincided with NFκB DNA binding activity, suggesting that it might indeed be one of the downstream effector genes of activated NFκB in vivo in the mammary gland.

MIS Induces NFκB DNA Binding Activity and IEX-1S mRNA in Mammary Glands of Mice in Vivo—Because MIS type II receptor levels, NFκB DNA binding activity, and IEX-1S expression demonstrated a compelling correlation during postnatal breast morphogenesis, we analyzed whether exogenous rhMIS could induce NFκB DNA binding activity and IEX-1S mRNA in the mammary glands of mice in vivo (n = 3). Exposure of mammary tissue to MIS resulted in the induction of NFκB DNA binding activity (Fig. 3A, top panel). Analysis of DNA-protein complexes demonstrated the presence of both p50 and p65 subunits; c-Rel was not present in the complex. The specificity of NFκB induction in vivo was demonstrated by incubating nuclear protein lysates with an oligonucleotide specific for OCT-1 (Fig. 3A, bottom panel). These experiments identify MIS as one of the first ligands that can induce NFκB DNA binding activity in the mammary gland in vivo. The levels of circulating rhMIS in the injected animals were estimated to be 2–4 μg/ml by MIS–enzyme-linked immunosorbent assay (2).

To determine whether MIS-mediated induction of NFκB DNA binding in vivo correlated with gly96/IEX-1 induction, Northern blot analysis was performed. Exogenous rhMIS in-
duced gly96/IEX-1 expression in the mammary tissue of mice in vivo. A, mammary glands of 8-week-old female mice were harvested 2 h after intraperitoneal injections of either 100 μg of MIS/animal or the same volume of vehicle (phosphate-buffered saline). Nuclear proteins were analyzed using a radiolabeled NFκB oligonucleotide probe. Positions of the DNA-NFκB protein complexes (closed arrow) and the supershifted complexes (open arrows) are indicated. Gel shift analysis performed with OCT-1 oligonucleotide is shown as control. B, 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 gly96/IEX-1 expression. RNA isolated from mammary glands of mice 6 h after intraperitoneal injection of PBS was used as control. Hybridization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown to control for loading. Bottom panel, cDNA derived from RNA isolated from mammary glands exposed to MIS for 6 h was analyzed using IEX-1-specific oligonucleotide primers. The presence of a single 296-base pair (bp) fragment revealed expression of gly96S/IEX-1 mRNA alone (closed arrow).

Fig. 4. MIS induces apoptosis of mammary epithelial cells in vivo. A, mammary tissue sections from 6-week-old female Rag2 mutant mice (n = 4) exposed to MIS or vehicle control (n = 4) were analyzed using fluorescein-labeled in situ cell death detection kit. Representative sections are shown at a magnification of × 60. B, the number of peroxidase-positive cells/duct in sections stained with ApopTag peroxidase apoptosis detection kit are represented as the ratio between apoptotic cells and ducts seen in mammary tissue exposed to MIS and normalized to vehicle-treated control. The unpaired Student’s t test demonstrated significant differences (p < 0.005) in apoptosis between mammary glands exposed to vehicle control and MIS.

**DISCUSSION**

Our previous demonstration that breast cancer cell lines express the MIS type II receptor and are a likely target for the growth inhibitory effect of MIS through a NFκB dependent
signaling pathway (10) were confirmed using MCF10A cells, a human breast epithelial cell line. MCF10A cells are immortalized but otherwise normal, and when seeded on Matrigel-coated plates, they form duct-like structures and secrete milk proteins into the lumen (1, 15).

Although development of the mammary gland begins in the embryo and continues after birth, the major developmental changes occur during puberty and pregnancy. At the onset of puberty, the terminal end buds rapidly proliferate, and the ducts elongate into the mammary fat pad (20, 24, 25). In 28-day-old Harlan Sprague-Dawley rats, most of the mammary fat pad is epithelium-free, and substantial ductal branching and invasion of the adipose stroma occurs by 50 days after birth (20). However, it is pregnancy that leads to complete maturation and functional activity of the breast with phases of proliferation, differentiation, and apoptosis during pregnancy, lactation, and involution (20, 24, 25). The inverse correlation between the abundance of MIS type II receptor mRNA expression and growth and functional differentiation of the breast during puberty, and at lactation and involution, suggests that MIS may mediate a negative growth regulatory signaling pathway. This hypothesis is supported by MIS-induced apoptosis in the breast epithelial cell compartment in vivo, which also confirms the induction of apoptosis observed in breast cancer cell lines in vitro. Mammary gland development during puberty occurs under the synergistic influence of growth hormone and estrogen (26), whereas progesterone and prolactin appear to be essential for glandular expansion during pregnancy and lactation. Whether repression of MIS type II receptor expression in the breast during pubertal changes and lactation could result from the action of these hormones provides an exciting area of future investigation.

The dynamic pattern of NFκB expression and activity in the breast epithelium during pregnancy, lactation, and involution (21, 22) and its aberrant DNA binding activity in breast cancer (27) suggest a role for this family of transcription factors in development and differentiation of the breast. Furthermore, a role for NFκB activation in functional differentiation of the breast epithelium is suggested by its ability to inhibit prolactin-induced STAT 5 phosphorylation, resulting in negative regulation of the β-casein gene expression (22). Brantley et al. (28) demonstrated that IκB-α is expressed uniformly in the cytoplasm of virgin, pregnant, and early lactating glands but decreases during late lactation and involution, which was ascribed to degradation resulting from NFκB activating signals. Targeted expression to the mammary glands of mice of a luciferase construct driven by a NFκB-responsive promoter demonstrated that luciferase expression strongly correlated with the oscillating NFκB DNA binding activity observed during postnatal breast morphogenesis. Thus, the NFκB DNA complexes activated during mammary gland development appear to be transcriptionally active (28). Little is understood about how transcription factors, such as NFκB, are influenced by extracellular signals and local growth factors known to affect breast development in vivo or which downstream effector genes are induced as a result of NFκB activation. Our studies identify MIS as an in vivo hormonal activator of this transcription factor in the breast.

The homo- or heterodimers arising from the NFκB family of transcriptional activators p65, p50, p52, and c-Rel have been shown to display differences in DNA binding specificity (29). Furthermore, transcriptional activation of genes involved in Drosophila immunity is differentially regulated by Rel-related protein dimers (29, 30). Interestingly, the NFκB complexes induced by MIS in breast cancer cell lines, MCF10A cells, and the mammary gland in vivo consisted predominantly of p50/p65 heterodimers, demonstrating a consistent cell-specific pattern of activation.

MIS-induced activation of NFκB in breast cancer cell lines resulted in up-regulation of the immediate early gene IEX-1S; overexpression of this gene inhibited breast cancer cell growth by 50% in colony inhibition assays, identifying IEX-1S as a putative NFκB inducible growth inhibitory gene in the breast (10). Arlt et al. (31) recently demonstrated that inducible expression of IEX-1/PRG1 in HeLa cells interferes with cell cycle progression and increases susceptibility to apoptosis. The correlation between NFκB DNA binding activity and PRG1S/IEX-1 expression in the mammary tissue of rats during pregnancy-related changes suggests that IEX-1S/PRG1S/gly96S may be one of the targets activated by NFκB nuclear localization in vivo. This is also suggested by the increase in NFκB DNA binding activity after exposure to MIS and the induction of IEX-1S/PRG1S/gly96S expression in human breast epithelial cells in vitro and in the mammary glands of mice in vivo. Thus, MIS may be an important normal in vivo hormonal signal that regulates the activity of this transcription factor and the expression of its downstream effectors, including IEX-1S/PRG1S/gly96S.

The lack of developmental abnormalities in mammary gland morphology and function in both MIS ligand and MIS type II receptor-null mice may be due to existence of redundant signals, such as local secretion of both transforming growth factor β1 and transforming growth factor β3, which induces apoptosis of the mammary epithelial cells (32–34). Moreover, the possibility that MIS knockout mice could exhibit delayed involution following the removal of suckling pups still remains to be investigated. These studies encourage further characterization of the effects of MIS-mediated signaling events in both normal and neoplastic breast and would require the generation of transgenic mice with targeted expression of MIS or its receptor to the breast.

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