Insulin-like Growth Factor-II Regulates PTEN Expression in the Mammary Gland

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The tumor suppressor PTEN is altered in many cancers, including breast cancer, but only a handful of factors are known to control its expression. PTEN plays a vital role in cell survival and proliferation by regulating Akt phosphorylation, a key component of the phosphatidylinositol 3 kinase (PI3K) pathway. Here we show that insulin-like growth factor-II (IGF-II), which signals through PI3K, regulates PTEN expression in the mammary gland. IGF-II injection into mouse mammary gland significantly increased PTEN expression. Transgenic IGF-II expression also increased mammary PTEN protein, leading to reductions in Akt phosphorylation, epithelial proliferation, and mammary morphogenesis. IGF-II induced PTEN promoter activity and protein levels and this involved the immediate early gene egr-1. Thus, we have identified a novel negative feedback loop within the PI3K pathway where IGF-II induces PTEN expression to modulate its physiologic effects.

PTEN‡ is emerging as the most frequently altered tumor suppressor gene other than p53 (1). PTEN is mutated in Cowden’s syndrome, a condition of familial cancer predisposition, and is frequently altered in a variety of spontaneous cancers including breast cancers (2–4). The loss of even one PTEN allele in mice leads to a high incidence of tumors in a variety of tissues (3, 5). Breast cancer in humans is associated with a loss of heterozygosity or mutation of the PTEN gene, and decreased PTEN expression has been associated with invasive breast cancer and poor prognosis (2, 6, 7). The principal activity of PTEN is to dephosphorylate a phospholipid second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3), produced by phosphatidylinositol 3 kinase (PI3K) (8, 9). PIP3 is the major activator of the cell survival kinase Akt (8, 9). Thus, negative regulation of the PI3K pathway by PTEN is critical, and the loss of PTEN function creates an environment conducive to tumorigenesis.

Despite the obvious importance of PTEN, only a handful of molecules are known to control its expression. Intracellular molecules reported to regulate PTEN transcription include p53 (10), peroxisome proliferator-activated receptor γ (11) and Egr-1 (12). These proteins induce PTEN promoter activity and putative binding sites for each have been identified in the PTEN promoter. Transforming growth factor β and progesterone have also been proposed to alter PTEN expression. Transforming growth factor β inhibited PTEN expression, but the nature of this regulation is unknown (13). Endometrial PTEN levels were higher during the secretory compared with the proliferative phase of the menstrual cycle implying an association with progesterone levels (14). Given the critical role of PTEN in the control of cell survival and proliferation, it stands to reason that extracellular factors such as hormones or growth factors should also influence PTEN expression.

Insulin-like growth factors (IGFs) are potent mitogens that impact development, are implicated as risk factors in breast cancer, and are overexpressed in human cancers (15, 16). IGF-I and IGF-II are produced by breast cancer cell lines (17, 18), and administration of IGFs to breast cancer cells promotes cell proliferation and inhibits apoptosis (19, 20). IGFs are known to mediate their cellular effects, at least in part, through the PI3K pathway (19). Here we demonstrate through biochemical, genetic, and molecular studies that IGF-II negatively regulates this pathway by increasing PTEN expression. Our results show that this PTEN regulation functions during mouse mammary development and that IGF-mediated PTEN regulation involves the immediate early gene egr-1.

MATERIALS AND METHODS

Mice, Tissue, and Serum Analyses—The generation of mouse mammary tumor virus (MMTV)-IGF-II mice has been reported (21). Mice were maintained following the guidelines of the Canadian Council on Animal Care. Whole mount, in situ hybridization, BrdUrd immunohistochemistry, serum progesterone, and 17-β-estradiol analyses were performed as previously described (22).

Mammary Tissue Manipulations—Administration of recombinant proteins to mammary glands involved injection of human IGF-II (rhIGF-II; Calbiochem, San Diego, CA) in phosphate-buffered saline with 0.1% bovine serum albumin or rhIGF-I (Calbiochem) and insulin (Sigma) in 10 mM acetic acid with 0.1% bovine serum albumin and 5 mM HCl. The 4th inguinal mammary glands were exposed surgically, and 10 μl containing 1 μg of rhIGF-II, 1 μg of rhIGF-I, and 10 μg of insulin or vehicle was injected. Elvax-40 pellets containing 300 ng of rhIGF-II or vehicle were generated (23) and respectively implanted into the 4th inguinal or contralateral mammary glands of 33-day-old wild type mice,

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**RESULTS AND DISCUSSION**

**IGF-II Injection Increases Mammary PTEN**—We analyzed whether recombinant IGF-II administered in an acute or chronic regimen altered Akt and PTEN levels in mouse mammary tissue. Local injection into the gland increased phosphorylated Akt levels in a dose-dependent manner (Fig. 1A). As expected, IGF-II administration resulted in rapid and sustained phosphorylation of insulin receptor substrate 1 (IRS-1) (Fig. 1B). Despite this, Akt phosphorylation was only transient, peaking around one hour post-injection and declining to pre-injection levels an hour later (Fig. 1, A and B). Concomitant with phosphorylated Akt decline was an increase in PTEN protein (Fig. 1, A and B). IGF-II did not activate MAPK pathways as Erks or p38 were not phosphorylated at 1 h post-injection, whereas epidermal growth factor injection provided a positive control for Erk/p38 activation (data not shown). PTEN mRNA levels also rose over time (Fig. 1, D and E). Chronic exposure to elevated IGF-II was achieved through implanting...
Fig. 3. Reduced mammary ductal morphogenesis in MMTV-IGF-II mice. Representative carmine-alum-stained wholemounts of wild type (A) and MMTV-IGF-II (B) transgenic mice at day 55 of development; size bars, 1 mm. C, quantification of ductal morphogenesis during mammary development. Representative wholemounts of wild type (D) and transgenic (E) 75-day-old mammary tissue. Arrowheads indicate TEBs; size bars, 400 µm. F, quantification of TEBs during mammary development. BrdUrd immunohistochemistry in wild type (G) and transgenic (H) tissue at day 55.
IGF-II Regulates PTEN Expression

To investigate the biological relevance of IGF-II-mediated PTEN induction in the mammary gland, we used MMTV-IGF-II mice that we have previously generated (21). We compared and found similar levels of type-I IGF (IGF-IR), insulin (Fig. 2A), and type-II IGF-II receptors (data not shown) in the wild type and transgenic mammary tissue. We then confirmed that IGF-IR was activated in transgenic tissue by assessing phosphorylation of IRS-1 in the developing mammary gland (Fig. 2A).

Similar to our above findings with acute and chronic IGF-II administration, we found a significant reduction in phosphorylated Akt levels (Fig. 2A, B), concomitant with a significant increase in PTEN protein levels (Fig. 2, A and C) in transgenic mammary tissue. Immunohistochemistry localized the reduced phosphorylated Akt and elevated PTEN protein to the transgenic mammary epithelium (data not shown).

IGF-II Inhibits Mammary Ductal Development—The PI3K/Akt/PTEN pathway is at the crux of cell survival, capable of influencing apoptosis, and more recently has been shown to regulate cell cycle progression (25, 26). Cyclin D1, a key component of cell cycle progression, is a downstream effector of Akt (25). Diminished levels of phosphorylated Akt permit cyclin D1 degradation (26). Cyclin D1 levels were significantly lower in the IGF-II overexpressing mammary tissue in 55 day-old mice (Fig. 2A). At this age, mouse mammary tissue is normally undergoing intense proliferation associated with epithelial ductal morphogenesis. To determine the effects of IGF-II-induced PTEN and resulting reduction in PI3K signaling, we assessed mammary morphogenesis in MMTV-IGF-II mice. Mammary ductal morphogenesis was significantly retarded in transgenic mice (Fig. 3, A–F) as is evident by a reduction in epithelial duct length, number of ducts, and the presence of terminal end buds in 75-day-old mice. Terminal end buds normally disappear by this age, and their presence indicates that lengthening of the ducts is not yet complete (27). Consistent with this, mammary epithelial proliferation was significantly inhibited as measured by BrdUrd incorporation (Fig. 3, G–I). These phenotypes are opposite to those reported for conditional mammary PTEN knockout mice that have excessive ductal branching and mammary epithelial proliferation (28). This highlights the biological impact PTEN levels have in mammary morphogenesis.

Because pubertal mammary development is dependent on 17β-estradiol and progesterone (29), we measured the serum levels of these hormones and found no difference between transgenic and wild type mice (data not shown). This indicates that retarded mammary development did not arise from altered ovarian function in MMTV-IGF-II mice. We next determined whether chronic IGF-II exposure would phenocopy mammary ductal retardation. A slow-release recombinant IGF-II pellet was implanted in the developing mammary gland of wild type mice while the contralateral gland received a control pellet. The IGF-II pellet retarded mammary ductal length by an average of 26% (data not shown). These data show that ectopic IGF-II expression decreased epithelial proliferation and inhibited pubertal mammary morphogenesis.

Other well established signaling pathways linked to cell proliferation include Erk1/Erk2, p38 MAPK, and JNK/SAPK. However, no significant differences were found in the levels of phosphorylated Erk1/Erk2 or p38 MAPK in transgenic mammary tissue at day 55 (Fig. 3J). We assessed ATF-2, which is downstream of JNK/SAPK, and observed that ATF-2 phosphorylation was also unaltered (Fig. 3J). Thus, we ruled out the involvement of these potential pathways in mediating the inhibitory effect of IGF-II on epithelial proliferation.

55 of development; size bars, 75 μm. Arrowheads denote BrdUrd-positive epithelial cells. I, quantification of BrdUrd-positive cells. J, Western analysis of phosphorylated Erks, p38 MAPK, and JNK/SAPK. However, no significant differences were found in the levels of phosphorylated Erk1/Erk2 or p38 MAPK in transgenic mammary tissue at day 55 (Fig. 3J). We assessed ATF-2, which is downstream of JNK/SAPK, and observed that ATF-2 phosphorylation was also unaltered (Fig. 3J). Thus, we ruled out the involvement of these potential pathways in mediating the inhibitory effect of IGF-II on epithelial proliferation.
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IGF-II Effect Is Epithelial Cell Autonomous—Both epithelial and stromal factors influence ductal development (23, 30). We found that transgenic mammary IGF-II expression was exclusive to epithelial cells (Fig. 3, K and L). To determine whether epithelial IGF-II was sufficient to delay ductal progression, reciprocal mammary transplants were performed between wild type and transgenic mice. Cleared mammary fat pads were generated in both wild type and transgenic mice by severing the epithelial ductal trees prior to puberty. Transgenic mammary epithelium was implanted into wild type cleared fat pads (Fig. 3N), whereas wild type tissue was implanted into cleared transgenic fat pads (Fig. 3M). Epithelial ducts emanating from the transgenic transplants were significantly shorter than those from wild type transplants (Fig. 3O). Thus, this phenotype was epithelial cell autonomous; epithelial IGF-II rather than the host environment (stromal and endocrine factors) was responsible for the retarded mammary development. Because human breast cancer originates in epithelial cells, the existence of an IGF-II/PTEN link in epithelial cells and its relation to mammary growth is particularly relevant.

IGF-II Regulates PTEN Transcription via Egr-1—We first tested the requirement of PTEN in IGF-II mediated Akt inactivation. Akt phosphorylation was sustained upon IGF-II treatment in pten−/− MEFs in relation to the wild type MEFs. Additionally, the basal level of Akt activation was substantially higher in pten−/− MEFs (Fig. 4A). To determine whether IGF-II regulates PTEN transcription, we used a PTEN-luciferase construct, pPTEN. MEFs transfected with this construct showed significant induction of luciferase activity after IGF-II treatment compared with a control PGL3 vector (Fig. 4B), demonstrating that IGF-II is able to induce PTEN promoter activity. A similar increase in PTEN promoter activity was also observed in NIH3T3 cells (data not shown). The IGF axis is able to induce the transcription factor egr-1 in embryonic and cardiac fibroblasts (31, 32), and we have recently shown that egr-1 directly activates PTEN during irradiation-induced signaling to a similar magnitude as observed above (12). Therefore, we investigated the requirement of egr-1 for IGF-II regulation of PTEN transcription. MEFs transfected with PTEN-luciferase constructs lacking the three putative egr-1 binding sites (d117), but retaining the p53 binding site, failed to show luciferase induction following IGF-II treatment (Fig. 4B). Furthermore, egr-1−/− MEFs containing intact PTEN promoter constructs also showed no induction of luciferase activity when treated with IGF-II (Fig. 4B). The higher basal reporter activity in wild type cells may be reflective of its higher p53 levels compared with the egr-1−/− cells. In addition, IGF-II administration resulted in elevated PTEN protein levels in wild type MEFs but not egr-1 null MEFs (Fig. 4C). Furthermore, we found that IGF-II injection into the mouse mammary gland induced Egr-1 protein expression in vivo (Fig. 4D). At present, the receptors that mediate the IGF-II effect remain to be elucidated.

In this study we demonstrate that the growth factor IGF-II induces expression of the tumor suppressor gene PTEN. PTEN is the major negative regulator of PI3K signaling, the very pathway used by IGFs to transmit their growth-stimulatory signal. This represents the first example of a negative feedback loop in IGF signaling that operates through PTEN to control proliferation. The biological consequences of this feedback are illustrated by experimentally increasing IGF-II levels, which lead to reduced proliferation and delayed mammary development. Regulation of PTEN occurs at the transcriptional level and the immediate early gene egr-1 is a necessary component of this loop. Similar feedback loops have been demonstrated in other signaling pathways including signaling from the insulin receptor (33, 34).

The canonical IGF-II pathway is mitogenic and implicated in mammary carcinogenesis. Our study demonstrates that through up-regulation of PTEN, IGF-II exerts a hypomorphic effect during mammary gland development. This effect bears a striking similarity to the MMTV-neu mouse, a widely used model of mammary tumorigenesis that also has hypomorphic mammary glands (35). Our findings provide mechanistic insights into the complexity of oncogene action, whereby a growth factor can restrain its own mitogenic action by up-regulating a key tumor suppressor. Loss of this negative feedback loop may release the oncogene’s cancer promoting ability, allowing its proliferative effect to dominate.

We have found that the immediate early gene egr-1 is vital to the induction of PTEN by IGF-II. Studies have shown that egr-1 is an integral player in the IGF axis. It induces IGF-II promoter expression in HepG2 cells in response to hypoxia (36). Also, stimulation of IGF signaling induces egr-1 expression, an effect that may be dependent on IRS-1 (31, 32). Thus, egr-1 is both upstream and downstream of IGF-II signaling. It is conceivable that another level of regulation exists within the IGF-II/PTEN negative feedback loop wherein IGF-II signals through egr-1 to induce PTEN, but egr-1 also leads to IGF-II induction. Such multiple negative and positive regulatory loops likely operate within all cells to temper the actions of external growth stimuli.

We have previously examined the PI3K/Akt/PTEN pathway at a different stage of mammary physiology in MMTV-IGF-II mice, namely post-lactation involution. We found that, unlike during mammary development, IGF-II did not influence PTEN expression during mammary involution (21). This finding is not surprising because the two stages have diurnally opposed cell fates and are structurally, hormonally, and functionally distinct. The developing mammary gland is primarily composed of ductal epithelium proliferating under the influence of estrogen and progesterone, whereas involuting mammary gland is lobulo-alveolar epithelium undergoing apoptosis initiated by prolactin withdrawal (37, 38). The marked differences at these stages likely account for the disparate effects of IGF-II on PTEN.

Egr-1 and PTEN, the components of our proposed negative feedback loop of IGF-II signaling, have been individually implicated in breast cancer (2, 6, 7, 39). It is possible that alterations in egr-1 and/or PTEN break the negative feedback loop, allowing the proliferative effects of IGF-II to dominate. It is important to note that loss of PTEN function in breast cancer is predominantly through loss of expression and not mutation. Our findings describe a potentially important PTEN regulatory pathway involving the breast cancer mitogen, IGF-II.

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