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Estrogen and Myc Negatively Regulate Expression of the EphA2 Tyrosine Kinase

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Abstract Estrogen receptor and c-Myc are frequently overexpressed during breast cancer progression but are downregulated in many aggressive forms of the disease. High levels of the EphA2 tyrosine kinase are consistently found in the most aggressive breast cancer cells, and EphA2 overexpression can increase metastatic potential. We demonstrate, herein, that estrogen and Myc negatively regulate EphA2 expression in mammary epithelial cells. These data reveal EphA2 as a downstream target of estrogen and Myc and suggest a mechanism by which estrogen and Myc may regulate breast cancer. J. Cell. Biochem. 85: 714–720, 2002. © 2002 Wiley-Liss, Inc.

Key words: EphA2; Myc; estrogen; metastasis; estrogen receptor; anti-estrogens; breast cancer

Epidemiological, therapeutic, and experimental studies have linked estrogen to breast cancer initiation and progression [Dickson et al., 1989; Hulka et al., 1994; Rochefort, 1995; MacGregor and Jordan, 1998]. This understanding formed the basis for the clinical application of anti-estrogens, which compete with estrogen for binding to the estrogen receptor (ER) and block hormone-responsive breast cancers [Jordan et al., 1978]. Anti-estrogens can also decrease breast cancer incidence in certain high-risk populations [Osborne, 1999; Jordan, 2000; Yardley, 2000].

In contrast to the view of estrogen induction of breast cancer, accumulating evidence suggests that estrogen negatively regulates breast cancer metastasis. For example, ER-positive breast tumor cells are generally more differentiated and have lower metastatic potential than ER-deficient cells [Osborne et al., 1985; Price et al., 1990]. Moreover, experimental activation of ER in ER-deficient breast cancer cells decreases metastatic potential [Jiang and Jordan, 1992; Zajchowski et al., 1993; Levenson and Jordan, 1994]. Despite the interest in estrogen and anti-estrogens in cancer treatment and prevention, studies of ER signaling have not resolved how ER might negatively regulate breast cancer metastasis [Rochefort, 1995; Dubik et al., 1996; Katzenellenbogen et al., 2000].

Our laboratory has been studying the regulation of protein tyrosine kinase activity in normal and malignant human mammary epithelial cells [Kinch et al., 1995]. Our recent studies have focused upon one particular receptor tyrosine kinase, EphA2, which is overexpressed and functionally altered in a large number of breast cancer cell models and clinical specimens [Zantek et al., 1999, 2001]. We recently showed that EphA2 overexpression is sufficient to confer tumorigenic and metastatic potential upon non-transformed mammary epithelial cells [Zelinski et al., 2001]. However, the mechanisms that regulate EphA2 in mammary epithelial cells remain largely unknown. Herein, we show that EphA2 expression is negatively regulated by estrogen and c-Myc.

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MATERIALS AND METHODS

Cells and Antibodies

All mammary epithelial cells were cultured as described [Zelinski et al., 2001]. Antibodies for β-catenin and EphA2 (D7) were purchased from Upstate Biologicals, Inc. (Lake Placid, NY). Paxillin-specific antibodies were the gift from Dr. K. Burridge (University of North Carolina).

Western Blot Analysis

All Western blot analyses were performed as detailed [Zelinski et al., 2001]. Multiple EphA2-specific antibodies (clones B2D6 and D7; Upstate Biotech Inc.) (EphA2 polyclonal sera; Santa Cruz Biotech, Santa Cruz, CA) were utilized as confirmation. Sample collection, immunoprecipitation, and Western blotting were performed as described [Kinch et al., 1995]. All samples were normalized for total protein, and blots were stripped and re-probed with β-catenin antibodies (Transduction Laboratories, Lexington, KY) to control for equal loading.

Estrogen Treatment

MCF-10A1 cells were grown overnight prior to addition of the stated concentrations of hormones at 37°C for the times indicated. 17-β-estradiol, hydrocortisone, and tamoxifen were prepared as specified by the manufacturer (Sigma). All solutions were prepared to ensure that the final concentration of vehicle did not exceed 0.5%, which was used as a negative control for all experiments.

Northern Blot Analysis

Northern blot analyses were performed as described [Peters et al., 1997]. Briefly, 20 μg of total RNA was resolved on a 1% agarose–formaldehyde gel and transferred to nylon membranes (Nytran, Schleicher & Schuell). Radiolabeled DNA fragments representing the 3′UTR of human *EphA2*, human *c-myc*, or *G3PDH* (Clonetech, Palo Alto, CA) mRNAs were hybridized with the membranes and washed prior to autoradiography. The relative mobilities of 18S and 28S rRNA were visualized by ethidium bromide staining.

Transfection

C3H10T½ cells (ATCC CCL226) were stably transfected using calcium phosphate [Sambrook et al., 1989]. Transfections of HBL100, MCF-10A, BT474, and SKBR3 utilized Lipofectamine Plus™ according to manufacturer instructions (GIBCO-BRL). Cells were selected in 400 μg/ml G418 (Cellgro™, Mediatech Inc., Herndon, VA). The following plasmids were used: pRc/CMV566 or pcDNA3 neomycin resistance vectors (Invitrogen Corp., Carlsbad, CA), pMC29*v-myc* [Heaney et al., 1986], pM21*myc*, pRc/CMV566 ΔBmax, and pRc/CMV566 Amax + C [Krylov et al., 1997]. Co-transfections were performed with vectors encoding for neomycin resistance at a 1:10 ratio.

RESULTS

The levels of EphA2 protein were evaluated by Western blot analyses using a panel of cell models derived from either benign or neoplastic mammary tissues (Fig. 1A). These models were selected in part based on their well-characterized status of ER expression [Bae et al., 1993; Vladusic et al., 2000]. Nontransformed epithelial cells (MCF-10A1, MCF-A2, MCF-12A), which have relatively low levels of ER [Vladusic et al., 2000], expressed moderate levels of EphA2, whereas tumor-derived cells that overexpress ER (T47D, ZR-75-1, MCF-7) had undetectable levels of EphA2. The highest levels of EphA2 were consistently found in ER-deficient breast tumor cells (Hs578T, MDA-MB-435, MDA-MB-231, BT549). Two exceptions, BT474 and SKBR3 cells, did not express either ERα or EphA2 (Fig. 1, lanes 7 and 8). This observation ultimately was instructive for understanding a mechanism of EphA2 regulation as detailed below.

Based on the inverse relationship between cellular EphA2 protein levels and ER expression, we asked whether estrogen negatively regulates EphA2 expression (Fig. 1B). Estradiol treatment of nontransformed mammary epithelial cells decreased EphA2 protein levels and in a dose-dependent manner. The decrease in EphA2 was reproduced using multiple cell models (MCF-10A1, MCF-12A, MCF-A2) and was observed using concentrations of estradiol that were physiologically relevant to estrogen levels in pre-menopausal women (0.1–2 nM) (Fig. 1B, data not shown). Estrogen-mediated inhibition of EphA2 expression was rapid and reversible. Lower levels of EphA2 were observed within 4 h of estrogen treatment, and EphA2 remained at low levels for at least 24 h (Fig. 2A). EphA2 levels began to increase within 1 h after the removal of estradiol, and EphA2

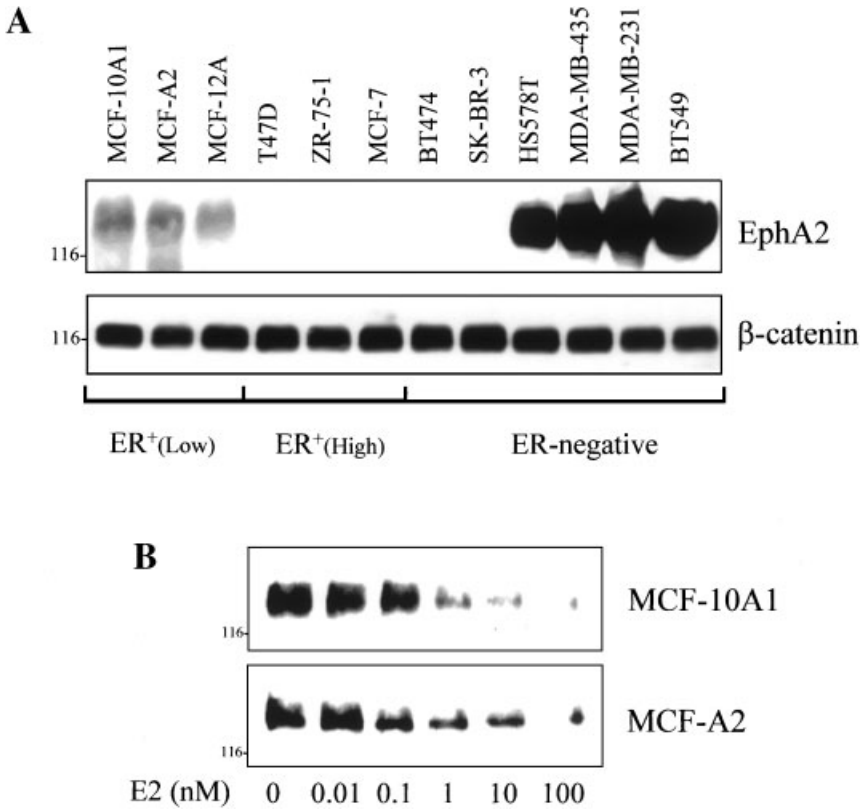

Fig. 1. Estrogen negatively regulates EphA2 expression. **A**: Whole cell lysates (10 μg) collected from nontransformed (**lanes 1–3**) and tumor-derived (**lanes 4–12**) mammary epithelial cells were subjected to Western blot analyses using EphA2-specific antibodies. The cell models were divided into three groups based upon their ER status (see text). The membranes were stripped and reprobed with antibodies specific for β-catenin to confirm equal protein loading. **B**: Equal numbers of MCF-10A1 or MCF-A2 cells were treated with the indicated concentrations of 17-β-estradiol (E2) for 12 h at 37°C before sample extraction and Western blot analyses. Note that the samples in (B) were overexposed relative to (A) to demonstrate the dose-dependence of estrogen-mediated inhibition of EphA2.

levels were restored to basal levels within 16 h (Fig. 2B).

Multiple controls for specificity confirmed that estradiol negatively regulates EphA2 expression. For example, EphA2 did not change in response to other steroid hormones, including cortisol or progesterone (Fig. 3A, data not shown). Moreover, the anti-estrogen tamoxifen prevented estradiol from decreasing EphA2 expression and in a dose-dependent manner (Fig. 3B). However, treatment of MCF-10A1 cells with 0–10 μM tamoxifen alone did not decrease EphA2 expression (Fig. 3C). Consistent with our findings, this anti-estrogen somewhat increased EphA2 levels, presumably by inhibiting residual estrogen activity provided by the cell culture medium. Finally, estradiol treatment of ER-deficient MDA-MB-231 breast cancer cells did not significantly alter EphA2 levels (Fig. 3D).

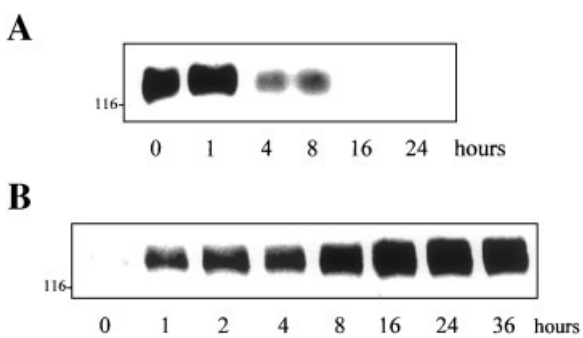

Fig. 2. Time-dependent and reversible inhibition of EphA2 by estrogen. MCF-10A1 cells were incubated with 5 nM 17-β-estradiol for the times indicated (**A**) or were incubated in culture media supplemented with 5 nM 17-β-estradiol for 6 h before replacing the culture medium with hormone-deficient media for the times shown (**B**). Cell lysates were resolved and submitted to Western blot analyses with EphA2 specific antibodies. Note that a longer exposure was utilized here relative to Figure 1A to optimize the analyses of EphA2 protein levels.

Recent studies by our laboratory and others have shown that EphA2 is overexpressed in Ras-transformed cells and that EphA2 overexpression increases metastatic potential [Walker-Daniels et al., 1999; Zantek et al., 1999; Zelinski et al., 2001]. To ask whether malignant transformation of ER positive mammary epithelial cells might inhibit the responsiveness of EphA2 expression to estrogen treatment, we examined MCF-10A1 cells that had been transformed by either oncogenic Ras

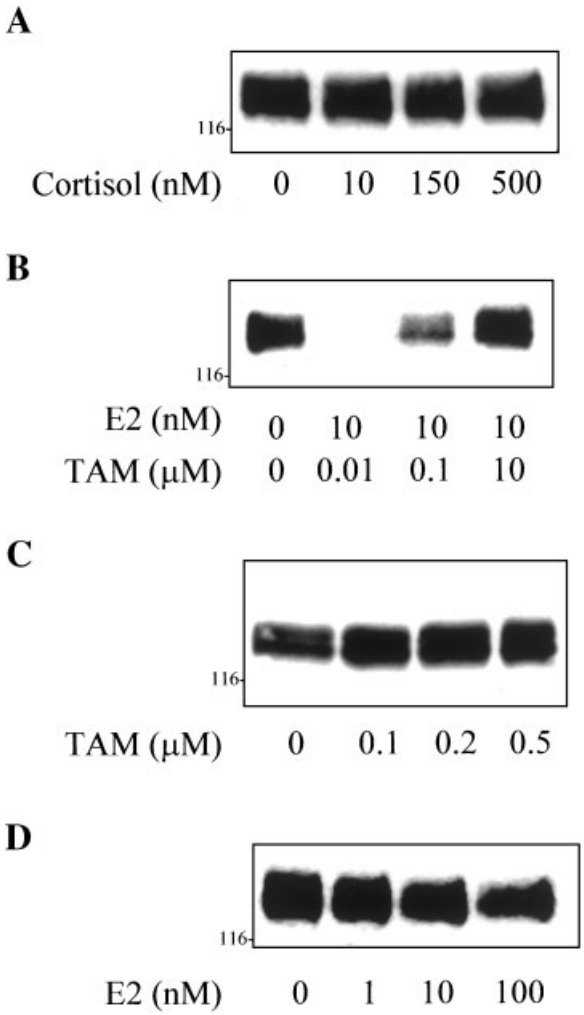

Fig. 3. Specificity of estrogen inhibition of EphA2. **A**: MCF-10A1 cells were treated for 18 h with the shown concentrations of cortisol. **B**: MCF-10A1 cells were incubated in the indicated amounts of 17-β-estradiol (E2) and tamoxifen (TAM) for 6 h. **C**: MCF-10A1 cells were incubated in media supplemented with the indicated amounts of tamoxifen for 18 h. **D**: ER-deficient MDA-MB-231 cells were incubated for 18 h in the shown concentrations of 17-β-estradiol. Cell lysates equalized for total protein concentration from each experiment were resolved by SDS–PAGE and analyzed by Western blot analyses with EphA2 specific antibodies.

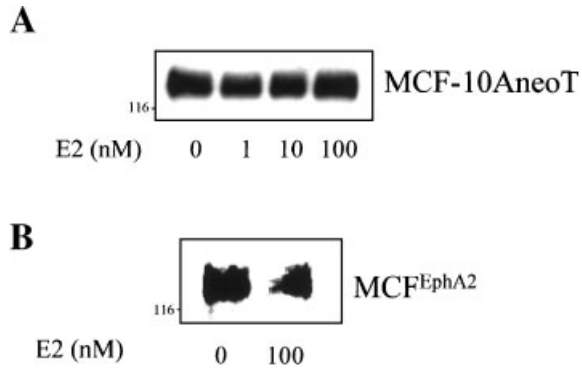

Fig. 4. Estrogen insensitivity of EphA2 in oncogenically-transformed mammary epithelial cells. MCF-10A1 cells that had been transformed by (**A**) oncogenic Ras (MCF-10neoT) or by (**B**) ectopic overexpression of EphA2 (MCF^{EphA2}) were treated for 18 h in the presence of the indicated amounts of 17-β-estradiol (E2). Cell lysates were analyzed by Western blot analyses using EphA2 specific antibodies. Note that the EphA2 in the transformed cells is not decreased in response to estrogen treatment.

(MCF-10AneoT) or EphA2 (MCF^{EphA2}). Cells were treated with estrogen (Fig. 4A,B), and in each case, EphA2 protein levels were minimally altered in response to estradiol. This finding indicates that EphA2 expression in oncogene-transformed breast epithelial cells is largely insensitive to estradiol.

BT474 and SKBR3 cells were unique in that they demonstrated low levels of both EphA2 and ER. c-Myc is one gene that is induced by estrogen [Dubik and Shiu, 1992], and we postulated that BT474 or SKBR3 might have elevated levels of Myc independent of ER. The levels of *ephA2* and c-*myc* mRNAs were evaluated using Northern blot analyses (Fig. 5A), which revealed an inverse relationship between c-*myc* and *EphA2* expression. Interestingly, the highest levels of c-*myc* mRNA were found in BT474 and SKBR3 cells, which had the lowest levels of *ephA2* mRNA.

Two sets of experiments provided more direct evidence that Myc regulates EphA2 expression. First, we overexpressed c-Myc in MCF-10A1 cells, which have low levels of endogenous Myc (not shown). Western blot analyses of cell lysates revealed that c-Myc overexpression was sufficient to downregulate EphA2 (Fig. 5B). A similar decrease in EphA2 was observed using multiple and different cell models, including HBL-100 and C3H-10T½ cells. We sought to perform comparable studies using ER-deficient breast cancer cells (e.g., MDA-MB-231, MDA-MB-435). However, overexpression of c-Myc induced considerable toxicities that precluded a reliable interpretation of data obtained from these particular experiments (data not shown).

In the inverse experiment, we inhibited c-Myc transcriptional activity in SKBR3 and BT474 cells, which express high levels of endogenous c-Myc. The endogenous c-Myc was inhibited by ectopic overexpression of dominant-negative forms of the B-max transcriptional regulator

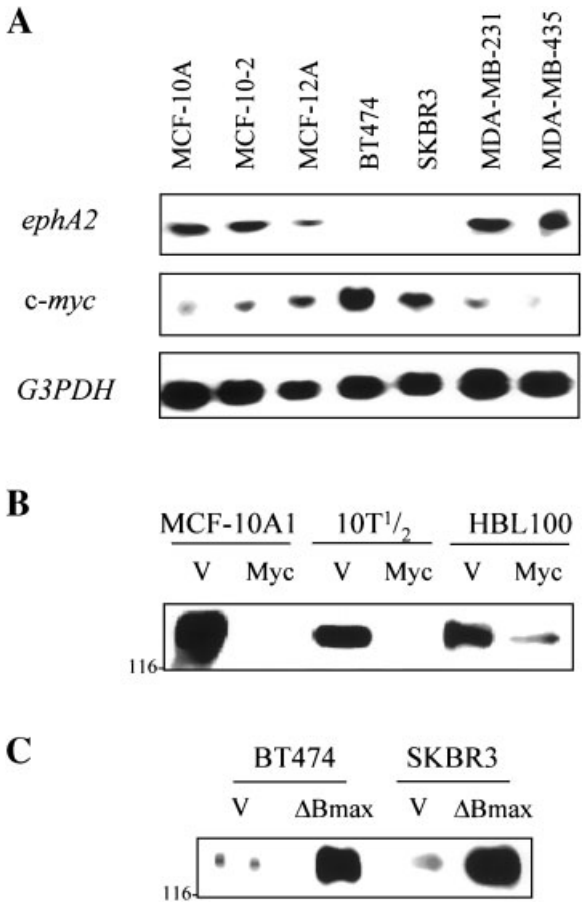

Fig. 5. Negative regulation of EphA2 expression by c-Myc. **A**: *ephA2* mRNA levels were evaluated by Northern blot analyses. The blots were stripped and reprobed for c-*myc* and then *G3PDH* as a loading control. Note the inverse correlation between *ephA2* and *c-myc* levels. **B**: EphA2 protein levels were measured in MCF-10A1, C3H-10T½, and HBL-100 cells following ectopic overexpression of c-Myc. All results are compared with parallel samples that had been transfected with a vector (V) control (CMV566). **C**: EphA2 protein levels were measured in BT474 or SKBR3 cells that had been transiently transfected with dominant negative inhibitors of c-Myc (ΔBmax) [Krylov et al., 1997] or vector-transfected controls (V). For all experiments, equal amounts of total cell proteins were loaded, and equivalent loading was confirmed by Western blot analyses with β-catenin-specific antibodies (not shown)

(ΔBmax, A-Max). Both mutant forms of the Max protein form nonproductive heterodimers with c-Myc, and overexpression of these constructs has been shown to inhibit endogenous c-Myc [Krylov et al., 1997]. Notably, overexpression of either ΔBmax or A-Max in either SKBR3 or BT474 was sufficient to increase EphA2 expression (Fig. 5C, data not shown). Thus, consistent results with multiple and different regulators of c-Myc function confirm that high levels of c-Myc negatively regulate EphA2 expression.

DISCUSSION

The major finding of our present study is that estrogen negatively regulates EphA2 expression in nontransformed breast epithelial cells at levels of estradiol that are relevant to premenopausal women. Decreased levels of EphA2 are pertinent to growth regulation since the EphA2 in nontransformed epithelial cells binds to its ligands, and thereby, transmits signals that negatively regulate cell growth [Zantek et al., 1999; Miao et al., 2000; Zelinski et al., 2001]. Our finding that estrogen decreases EphA2 expression suggests a mechanism by which estrogen may promote mammary epithelial cell growth.

The expression of ER-alpha is frequently lost in many of the most aggressive breast cancers [Jordan et al., 1978; Dickson et al., 1992; Jiang and Jordan, 1992; Zajchowski et al., 1993; Levenson and Jordan, 1994]. In light of our present findings, it is tempting to speculate that ERα expression might suppress EphA2 expression, and thereby, negatively regulate a metastatic phenotype. In contrast, ER-negative breast cancer cells express high levels of EphA2 protein, which promotes a metastatic phenotype. This idea is intriguing given that recent findings suggest anti-estrogens, such as tamoxifen, may not effectively treat the most aggressive (and often ER-negative) forms of the disease [Osborne, 1999; Jordan, 2000; Yardley, 2000]. Future investigation could, therefore, determine if or how EphA2 and ER relate to experimental and clinical metastasis.

The mechanism by which estrogen negatively regulates EphA2 expression is unknown, but our studies implicate a role for c-Myc. Estrogen is known to induce c-Myc expression within minutes [Santos et al., 1988; Shiu et al., 1993], which is consistent with the timing of EphA2 downregulation following estrogen treatment. The survival of the mammary epithelial cells studied here is highly dependent upon c-Myc function. Dominant-negative inhibitors of c-Myc induced apoptosis (not shown), which prevented us from determining whether c-Myc function is necessary for estrogen-mediated repression of EphA2 expression. Finally, while our studies link estrogen and c-Myc with EphA2 expression, we cannot exclude that other mechanisms of estrogen signaling (e.g., estrogen response elements in the EphA2 promoter, membrane signaling by E2) also contribute to

the negative regulation of EphA2 described herein.

One interesting aspect of EphA2 is its dual nature. In normal cells, EphA2 negatively regulates cell growth and invasiveness, whereas in malignant cells, EphA2 is overexpressed and functions as a powerful oncoprotein [Walker-Daniels et al., 1999; Zantek et al., 1999; Miao et al., 2000; Zelinski et al., 2001]. The mechanism responsible for these very different functions is the subject of intense investigation by our laboratory and others, and has been linked with the status of EphA2-ligand binding [Zantek et al., 1999; Miao et al., 2000]. Specifically, unstable cell–cell contacts prevent EphA2 from interacting with its ligands, which are anchored to the membrane of neighboring cells [Zantek et al., 1999]. Decreased EphA2-ligand binding promotes, rather than inhibits, cell growth and invasiveness [Zelinski et al., 2001]. Notably, EphA2 overexpression destabilizes cell–cell adhesions, and thereby, alters its own function. Thus, upregulation of EphA2 levels beyond a particular threshold could cause a growth-inhibitory signal to be modified so that, it instead, promotes malignant behavior. We suggest that loss of ER-mediated repression of EphA2 protein levels could, thereby, promote a metastasis by contributing to overexpression of and functional alterations in EphA2.

Our previous findings have also noted an interesting relationship between the ability of EphA2 to interact with its ligands and EphA2 protein stability [Zantek et al., 1999, 2001]. In addition to its affects on cell behavior (see above), another consequence of EphA2-ligand binding is EphA2 proteolysis [Walker Daniels and Kinch, unpublished communications]. As the EphA2 in malignant cells generally fails to bind ligand [Zantek et al., 1999], EphA2 protein is generally more stable in malignant cells than in nontransformed epithelial cells, where it does bind ligand. Thus, differential protein stability may explain why malignant breast cancer cells have higher levels of EphA2 protein than nontransformed mammary epithelial cells, despite comparable EphA2 mRNA expression (for an example, compare Figures 1A and 5A). Our present findings with estrogen and Myc suggest that EphA2 can also be regulated at the level of gene expression. Consistent with this, a recent study demonstrated that EphA2 gene expression is also negatively regulated by members of the p53 family of transcriptional regulatory proteins [Dohn et al., 2001]. Thus, the cellular levels of EphA2 protein are dictated by a complex regulation of both EphA2 gene expression and EphA2 protein stability.

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