A conditional feedback loop regulates Ras activity through EphA2

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Summary

The EphA2 receptor tyrosine kinase is frequently overexpressed in many cancers, including 40% of breast cancers. Here, we show that EphA2 is a direct transcriptional target of the Ras-Raf-MAPK pathway and that ligand-stimulated EphA2 attenuates the growth factor-induced activation of Ras. Thus, a negative feedback loop is created that regulates Ras activity. Interestingly, the expression of EphA2 and ephrin-A1 is mutually exclusive in a panel of 28 breast cancer cell lines. We show that the MAPK pathway inhibits ephrin-A1 expression, and the ligand expression inhibits EphA2 levels contributing to the receptor-ligand reciprocal expression pattern in these cell lines. Our results suggest that an escape from the negative effects of this interaction may be important in the development of cancer.

Introduction

Eph proteins form the largest family of receptor tyrosine kinases in the human genome, with 16 Eph receptors and nine ephrin ligands (Palmer and Klein, 2003).

Eph receptors and their ligands regulate axon guidance (Brittis et al., 2002), cell migration (Knoll and Drescher, 2002; McLennan and Krull, 2002; Sharfe et al., 2002; Wilkinson, 2000), morphogenesis (Miao et al., 2003), and vasculature (Bovenkamp and Greer, 2001; Ogawa et al., 2000).

There are two classes of ligands for Eph receptors: ephrin-A and ephrin-B. A-type ephrins are anchored to the plasma membrane via GPI linkage, whereas B-type ephrins have a transmembrane domain and an intracellular domain. Depending on which type of ephrin they interact with, Eph receptors are also classified as EphA or EphB. Upon ligand binding, Eph receptors dimerize and become phosphorylated (Dodelet and Pasquale, 2000).

Eph family members have been implicated in cellular transformation, metastasis, and angiogenesis (Nakamoto and Bergemann, 2002). EphA2 is overexpressed in a variety of cancers, including breast cancer (Zelinski et al., 2001), melanoma (Easty et al., 1995), and prostate cancer (Walker-Daniels et al., 1999).

EphA2 has also been shown to play a role in malignant transformation of MCF10A cells (Zelinski et al., 2001). In order to determine the role of EphA2 in the initiation and/or progression of cancer, it is important to understand how EphA2 levels are regulated.

Activation of the EphA2 receptor has been shown to stimulate the mitogen-activated protein kinase (MAPK) pathway (Pratt and Kinch, 2002) and the PI3K pathway (Pandey et al., 1994). In contrast, others have shown that activated EphA2 downregulates the Ras/MAPK pathway (Miao et al., 2001) via recruitment of p120GAP (Tong et al., 2003). A recent report suggests that ligand binding upregulates EphA2 mRNA through the MAPK pathway (Pratt and Kinch, 2003). Here, we demonstrate that EphA2 is a direct transcriptional target of the Ras-Raf-MAPK pathway and that ligand-stimulated EphA2 attenuates the growth factor-induced activation of this pathway. We propose that EphA2 signaling contributes to a feedback loop that regulates Ras activity in a ligand-dependent manner. We also show that EphA2 is expressed in a subset of breast cancer cell lines and that the expression of EphA2 and its cognate ligand, ephrin-A1, is inversely proportional in these cell lines. Two distinct mechanisms contribute to the inverse correlation between EphA2 receptor and ephrin-A1 ligand expression.

SIGNIFICANCE

Ras is frequently hyperactivated in human tumors. Our results reveal that Ras activity is controlled by a negative feedback loop through EphA2 in a ligand-dependent manner. Thus, deregulation of Eph signaling through downregulation of either receptor or ligand expression may contribute to hyperactive Ras and tumor development.

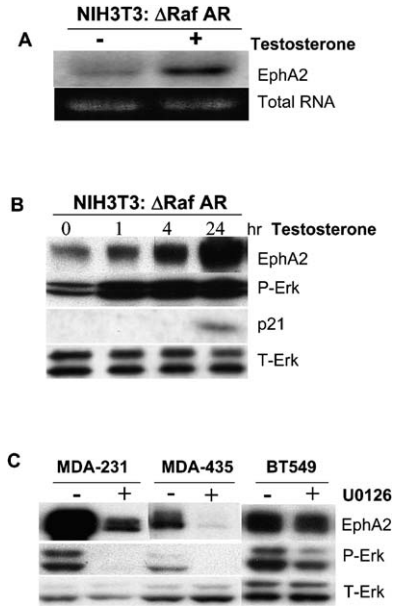

Figure 1. EphA2 is a transcriptional target of the Raf/MAPK pathway

A: Raf activation stimulates EphA2 mRNA. NIH3T3:ΔRafAR cells were serum starved for 32 hr prior to treatment with either 300 nM testosterone or ethanol for 24 hr. The Northern blot was probed with ^{32}P-labeled EphA2 cDNA. Total RNA served as the loading control.

B: Raf activation stimulates the EphA2 protein. Serum-starved NIH3T3: ΔRafAR cells were treated with either 300 nM testosterone for 1, 4, or 24 hr, or ethanol (labeled as 0). The Western blot was probed with an anti-EphA2 monoclonal antibody. Phospho-Erk and p21/Cip1 served as positive controls for Raf activation. Total Erk served as the loading control.

C: Inhibition of the MAPK pathway inhibits EphA2 levels. Breast cell lines were treated with either 10 μM MEK inhibitor U0126 (Promega) or DMSO for 24 hr. Cell lysates were analyzed for EphA2 protein expression. P-Erk served as a positive control for MEK activity, and T-Erk served as the loading control.

Results

Raf activation stimulates EphA2 mRNA

We analyzed transcriptional targets of the Raf-MEK-MAPK pathway using microarrays consisting of 15,552 mouse cDNA clones (Reynolds, 2002; VanBuren et al., 2002). RNA was generated from NIH3T3:ΔRafAR cells that express a fusion protein, comprised of an oncogenic form of human Raf-1 and the hormone binding domain of an androgen receptor. Upon androgen addition, Raf kinase activity and the MAP kinase pathway are activated (Samuels et al., 1993; Shelton et al., 2003).

Serum-starved NIH3T3:ΔRafAR cells were stimulated with 300 nM testosterone or ethanol for 24 hr. RNA was isolated, differentially labeled with cy3/cy5, and hybridized to the cDNA microarrays (DeRisi et al., 1996). The arrays were scanned and analyzed using GenePix 3.0 software. Results were compiled from three separate experiments, each performed in duplicate. In response to MAPK activation, EphA2 expression was stimulated 3.5- to 5-fold. Cyclin D1, a previously described downstream target of Raf, was stimulated 3-fold in the same experiment (data not shown). To validate the microarray results, we performed a Northern blot with RNA prepared from NIH3T3: ΔRafAR cells as described above and using EphA2 cDNA as probe. A single transcript was found to be stimulated upon treatment with testosterone (Figure 1A).

To test if the MAPK pathway directly activates transcription of *EphA2*, we treated NIH3T3 cells harboring Raf fusion construct described above with 20 μg/ml cycloheximide 15 min prior to stimulating Raf and measured EphA2 mRNA expression by quantitative RT-PCR. Cycloheximide did not prevent EphA2 mRNA induction, showing that transcriptional activation of *EphA2* by MAPK did not depend on new protein synthesis (data not shown).

Raf activation stimulates EphA2 protein levels

To determine if Raf activation also increases levels of EphA2 protein, serum-starved NIH3T3:ΔRafAR cells were stimulated as described above but for 1, 4, or 24 hr. Cell extracts were prepared and resolved on an SDS-PAGE gel, and protein levels were analyzed by Western blotting with a monoclonal anti-EphA2 antibody. EphA2 protein levels increased within 1 hr of Raf activation, with further increases at 4 and 24 hr time points (Figure 1B). As described previously, acute activation of Raf kinase also induced expression of p21/Cip1 (Figure 1B) and cyclin D1 (data not shown).

To rule out the possibility that activation of EphA2 by the MAPK pathway in NIH3T3:RafAR cells may be an effect of testosterone or unique to fibroblasts, we examined the effect of MAPK activation on EphA2 levels in immortalized but non-transformed human breast epithelial MCF10A:ΔRafER cells (Schulze et al., 2001). These cells express a RafER fusion protein that can be activated by addition of 4-hydroxy tamoxifen (Samuels et al., 1993). As expected, the hormone treatment induced Raf activity and EphA2 protein levels (Figure 5A). These results support our microarray and Northern data and indicate that EphA2 is a downstream target of the Raf/MAPK pathway.

EphA2 protein levels are inhibited by inhibition of MEK activity

To further confirm EphA2 as a target of the MAPK pathway, we examined EphA2 protein levels in MDA-MB231, MDA-MB435, BT549, and MCF10A breast cell lines in the presence or absence of MEK inhibitor U0126 (Promega). Cells were treated with either 10 μM U0126 or DMSO for 24 hr. EphA2 protein levels were analyzed by Western blotting with an anti-EphA2 antibody. As shown in Figures 1C and 5B, EphA2 protein levels were inhibited by U0126. Another well-characterized MEK inhibitor, PD 98059, had similar effect on EphA2 levels (Figure 5B). This confirms our results that EphA2 is a target of the Raf/MAPK pathway.

Ligand-stimulated EphA2 attenuates EGF-induced activation of the MAPK pathway

Eph-ephrin interaction results in the Eph receptor being phosphorylated (Dodelet and Pasquale, 2000). We noticed that this treatment downregulated P-Erk levels in HBL100 cells (data not shown). To characterize this further, we exposed serum-starved BT549, MDA-MB157, HBL100, MDA-MB231, and MCF10A cells to a soluble ephrin ligand, ephrin-A1/Fc, and measured its effects on ERK phosphorylation following EGF treatment. Figure 2 shows that, in most cases, the stimulation of ERK phosphorylation in response to EGF was reduced significantly following the activation of EphA2 by ephrin-A1 ligand (MCF10A data not shown). The exception was MDA-MB231: in these cells, the levels of P-Erk were independent of EGF and

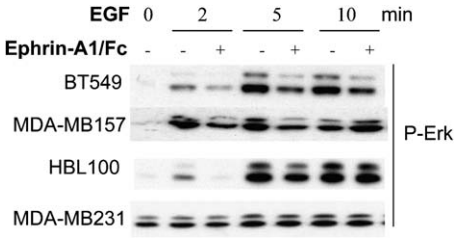

Figure 2. Ligand-mediated EphA2 stimulation attenuates the EGF-induced activation of the MAPK pathway

Cells were serum starved for 24 hr, pretreated with 1 μg/ml ephrin-A1/Fc for 5 min, and then stimulated with 10 ng/ml EGF for 2, 5, or 10 min (BT549 cells were stimulated with 1 ng/ml EGF). MAPK activity was assessed using a polyclonal anti-phospho-Erk antibody.

ephrin ligand, probably because this cell line harbors a mutation in *B-Raf* (Wilhelm et al., 2004) and an activated allele of *K-Ras* (Kozma et al., 1987; Ogata et al., 2001) that does not require EGF for activation and cannot be downregulated by the recruitment of p120GAP. Indeed, stable expression of H-RasV12 in BT549 cells that express normal levels of Ras renders them insensitive to inhibition of EGF-induced activation of P-Erk by ligand-stimulated EphA2 (data not shown).

EphA2 and ephrin-A1 expression is inversely proportional in breast cancer cell lines

We have shown that the MAPK pathway activates EphA2 expression and that ligand-stimulated EphA2 downregulates P-Erk levels. This prompted us to propose that EphA2 signaling may regulate Ras activity through a negative autocrine loop where ephrin ligand activates EphA2 cell autonomously. To explore this possibility, we examined the expression of EphA2 and its ligands in a panel of breast cancer cell lines in which gene expression profiles, gene copy number, and a number of biochemical properties have been examined comprehensively (K. Chin, R.M.N., F.M., and J.W.G., unpublished data). We found an inverse correlation between the expression of EphA2 receptor and ephrin-A1 ligand (Figure 3A). EphA2 protein was expressed in 8 of 28 cell lines tested. These eight cell lines express molecular markers of a mesenchyme-like phenotype: they fail to express E-cadherin, for example, and express high levels of vimentin (L. Timmerman, J.Y., J.W.G., and F.M., unpublished data). Each of these cell lines is ER negative (J. Gump and F.M., unpublished data), confirming their lack of breast epithelial characteristics (Sorlie et al., 2001). Cells expressing the highest level of EphA2 mRNA (MDA-MB231) also express a mutant, activated Ras and mutant B-Raf, confirming the relationship between MAPK activation and EphA2 expression.

In contrast, cells expressing the highest levels of ephrin-A1 ligand were those with epithelial characteristics (E-cadherin expression, no vimentin), such as the well-characterized SK-BR3 cells (L. Timmerman, J.Y., J.W.G., and F.M., unpublished data). EphA2 and ephrin-A1 protein levels corresponded well with mRNA levels (Figure 3B). Ephrin-A2 and -A5 were not expressed in these cells, while the pattern of ephrin-A3 and -A4 mRNA levels paralleled that of ephrin-A1 (Figure 3B). Thus, amongst this panel of breast cancer cell lines, none appear to express both ligand and receptor and therefore none maintains negative feedback of Ras signaling through EphA2 activation.

Ephrin-A1 suppresses EphA2 protein levels

Breast cancer cells that express ephrin-A ligands fail to express EphA2. Therefore, we addressed the possibility that, in these cells, ephrin ligand inhibits the expression of EphA2. Indeed, ephrin-A1 siRNA enhanced EphA2 protein levels significantly in SK-BR3 (Figure 4A) and BT474 cells (data not shown). On the other hand, ectopic expression of ephrin-A1 downregulated EphA2 protein levels in MDA-MB231 and BT549 cells (Figure 4B). Taken together, these results strongly indicate that the expression of ephrin-A1 suppresses EphA2 protein levels. Increased expression of EphA2 following knockdown of ligand expression is not due to activation of the MAPK pathway (levels of phospho-ERK do not change following ephrin knockdown; data not shown) and must involve yet another element of crosstalk and regulation.

One possible mechanism for downregulation of the EphA2 receptor by the ephrin ligand described above may involve ligand-mediated receptor internalization. To explore this possibility, we treated EphA2-expressing HBL100 cells with soluble ephrin-A1/Fc for 1, 3, or 7 hr and examined EphA2 localization by immunofluorescence. EphA2 staining was redistributed from plasma membrane within 1 hr after treatment with ephrin-A1/Fc, and decreased EphA2 staining was observed after longer exposures (Figure 4C). Similar results were obtained with MDA-MB231 and BT549 cells (data not shown). Treatment with Fc alone had no effect on EphA2 staining pattern (data not shown). We conclude that cells expressing the ephrin ligand suppress EphA2 expression, at least in part, through ligand-dependent receptor internalization (Figure 4C) and degradation (Walker-Daniels et al., 2002).

The MAPK pathway suppresses expression of ephrin-A1

We have shown that breast cancer cells that express EphA2 do not express ephrin-A1 ligand and that EphA2 expression in these cells is regulated, at least in part, by the MAPK pathway. We considered the possibility that ephrin-A1 ligand in these cells is suppressed by this pathway based on the recent discovery that active β-catenin both activates transcription of EphB receptor and downregulates ephrin-B ligand, to determine cell fate in the intestinal epithelium (Batlle et al., 2002). Indeed, the activation of the MAPK pathway in MCF10A: ΔRafER cells by 4-hydroxy tamoxifen inhibited levels of ephrin-A1 (Figure 5A), and the inhibition of MAPK activity by MEK inhibitors UO126 and PD 98059 increased ephrin-A1 levels (Figure 5B). We conclude that the lack of ephrin-A1 expression in our panel of breast cancer cell lines expressing the EphA2 receptor may be due, in part, to inhibition of ephrin-A1 expression by MAPK signaling.

Ephrin-A1 presented on neighboring cells can activate the EphA2 receptor

We have shown that the MAPK pathway both activates transcription of the EphA2 receptor and inhibits ephrin-A1 levels. Therefore, we reasoned that the ligand-induced activation of EphA2 is non-cell-autonomous and via ligand presented on neighboring cells such as the stromal cells. To address this possibility, we cocultured BT549 (receptor-expressing) and T47D (ligand-expressing) cells and examined tyrosine phosphorylation on EphA2 after immunoprecipitating the EphA2 receptor with an anti-EphA2 antibody. Treatment with soluble ephrin-A1/Fc served as a positive control. Indeed, EphA2 was

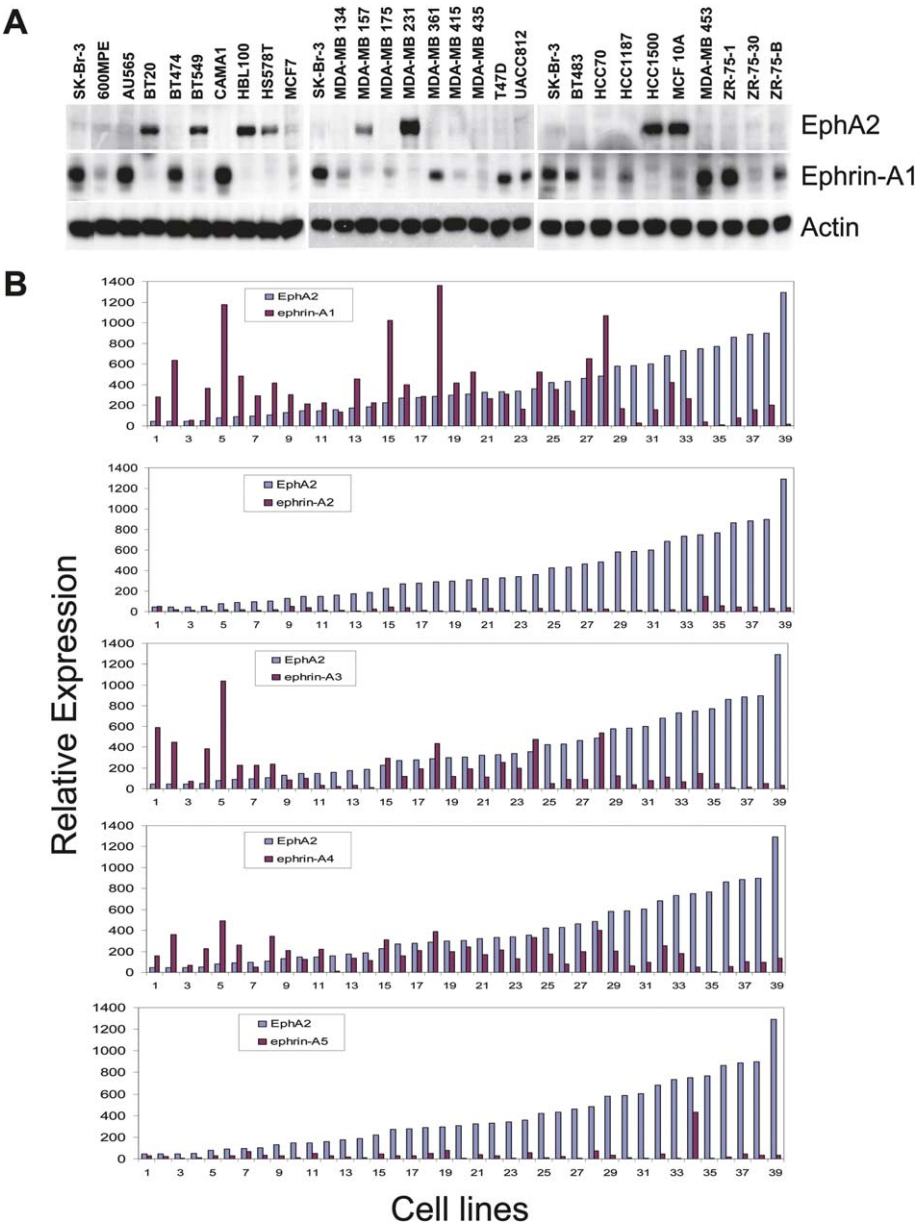

Figure 3. Expression of EphA2 and its ligand, ephrin-A1, is inversely proportional in breast cancer cell lines

A: Extracts were prepared from 28 different breast cell lines (note: HBL100 cell line is reported to have a Y chromosome). Ten micrograms of total protein was resolved on SDS-PAGE gels, transferred to PVDF membranes, and probed with either an anti-EphA2 or an anti-ephrin-A1 antibody. A monoclonal anti-actin antibody was used to ensure equal loading.

B: Affymetrix array analysis of EphA2 and ephrin-A mRNA expression in 39 breast cancer cell lines. Expression levels are represented by bar graphs. x axis, cell lines; y axis, relative expression; EphA2, blue color; ephrin-A, red color. Order of cell lines from left to right: 1, HCC1428; 2, BT483; 3, MDA-MB134; 4, T47D; 5, ZR75-1; 6, SUM-44PE; 7, MDA-MB361; 8, BT474; 9, MDA-MB453; 10, SUM-52PE; 11, MDA-MB435; 12, ZR75-30; 13, MDA-MB415; 14, HCC1569; 15, MCF7A; 16, LY2; 17, HCC1143; 18, HCC3153; 19, SK-BR3; 20, HCC1007; 21, HCC2185; 22, HCC1937; 23, HCC2157; 24, 600MPE; 25, MDA-MB436; 26, MDA-MB468; 27, HCC202; 28, BT549; 29, MDA-MB157; 30, HBL100; 31, BT20; 32, SUM-149PT; 33, ZR75B; 34, DU4475; 35, HS578T; 36, HCC38; 37, HCC1954; 38, MDA-MB231; 39, SUM-159PT.

tyrosine phosphorylated when presented with the ligand-expressing T47D cells (Figure 6), indicating that the inhibition of Ras activity by ligand-stimulated EphA2 is not an autocrine event, but rather occurs through binding to ligand presented on neighboring cells.

Ephrin expression inhibits transformation by v-ErbB in NIH3T3 cells

The ligand-stimulated EphA2 attenuates the EGF-induced activation of the Ras/MAPK pathway. This suggests that EphA2 signaling plays an important role in regulating Ras activity. We inquired if the ligand-induced activation of EphA2 inhibits transformation by v-ErbB, an event known to be dependent on Ras activation (McCubrey et al., 2004). The expression of ephrin-A1 suppressed v-ErbB transformation significantly, as determined by the reduction in colony formation in soft agar (Figure 7). In contrast, NIH3T3 cells transformed with H-RasV12 formed colonies at the same efficiency in the absence or presence of ephrin expression (data not shown). NIH3T3 cells expressing an empty vector had no colonies (data not shown). Taken together, these data are consistent with the role of EphA2 signaling in regulation of Ras activity, support our negative feedback loop model (Figure 8), and suggest a biological role of EphA2 signaling in tumorigenesis.

Discussion

A negative feedback loop regulates Ras activity through EphA2 in a ligand-dependent manner

Normal cells respond to growth factor stimulation by turning on the MAPK pathway, resulting in cell proliferation, cell differ-

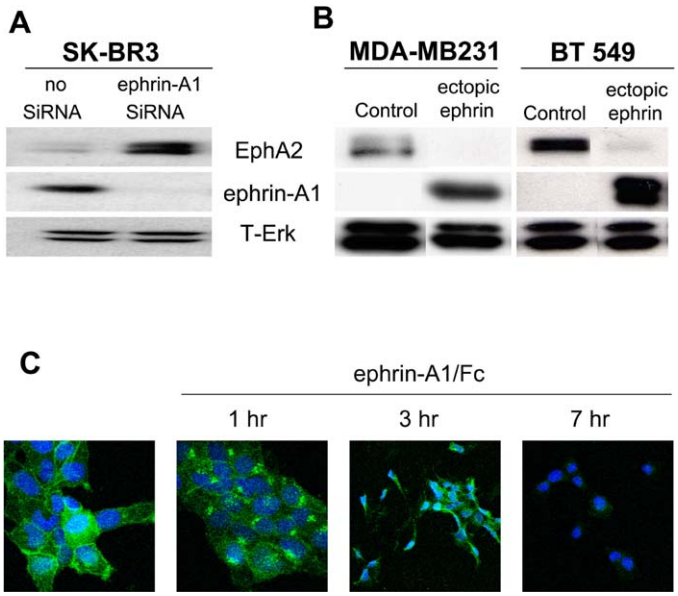

Figure 4. Ephrin-A1 suppresses EphA2 protein levels

A: SK-BR3 cells were transiently transfected with ephrin-A1 siRNA smartpool from Dharmacon. Cells were harvested 48 hr after transfection. Lysates were analyzed for EphA2 and ephrin-A1 protein expression. Total Erk served as the loading control.

B: MDA-MB231 and BT549 cells were stably transfected with either an empty vector or ephrin-A1 cDNA. Lysates were analyzed for EphA2 and ephrin-A1 protein expression. Total Erk served as the loading control.

C: HBL100 cells were grown in 8-well dishes and treated with 1 μg/ml soluble ephrin-A1/Fc for 1, 3, or 7 hr. Cells were fixed and labeled with a monoclonal anti-EphA2 antibody for 1 hr followed by Alexa 488 secondary antibody. Nuclei were counterstained with DAPI. Cells were visualized by confocal microscopy.

entiation, and other cellular processes. Hyperactivation or deregulation of this pathway is responsible for many human cancers. Mechanisms exist to maintain homeostasis in normal cells. Our results point to one such mechanism. We show that Raf activation stimulates EphA2 mRNA expression and protein

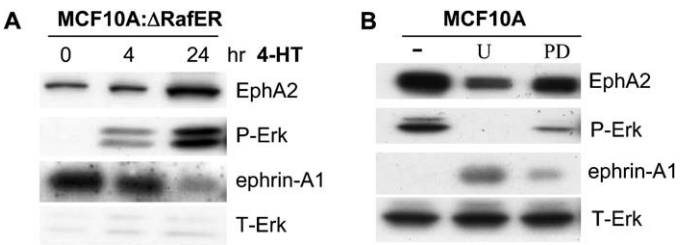

Figure 5. MAPK pathway activates transcription of *EphA2* and downregulates ephrin-A1 levels in breast epithelial cells

A: MCF10A:ΔRafER cells were serum starved for 32 hr prior to treatment with either 300 nM 4-hydroxy tamoxifen for 4 or 24 hr, or ethanol (labeled as 0). The Western blot was probed with an anti-EphA2 monoclonal antibody. Phospho-Erk served as the positive control for Raf activation, and total Erk served as the loading control.

B: MCF10A cells were treated with 10 μM MEK inhibitors U0126 (U) or PD 98059 (PD) for 24 hr. The Western blot was probed with anti-EphA2 and anti-ephrin-A1 antibodies. P-Erk served as a positive control for MEK activity, and total Erk served as the loading control.

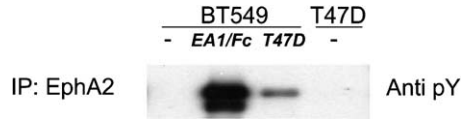

Figure 6. EphA2 receptor is phosphorylated by ephrin-A1 ligand presented on neighboring cells in coculture

Receptor-expressing BT549 cells were either incubated with soluble ephrin-A1/Fc (EA1/Fc) ligand or cocultured with ephrin-expressing T47D cells for 1 hr. Cells were lysed. The EphA2 protein was immunoprecipitated with an anti-EphA2 antibody and analyzed for phosphotyrosine content by Western blotting using a monoclonal anti-phosphotyrosine antibody (pY).

levels. This may account for high levels of EphA2 in many cancer cells, such as in malignant melanoma, in which the Ras-Raf-MAPK pathway is invariably activated, through activation of either Ras or B-Raf (Davies et al., 2002). We, and others, also show that EphA2 attenuates growth factor-induced activation of the MAPK pathway in a ligand-dependent manner, possibly through p120GAP. Taken together, these results indicate that the interplay between growth factor-induced Ras signaling and EphA2 receptor kinase signaling forms a conditional feedback loop that regulates Ras activity (Figure 8).

We explored the possibility that a negative autocrine loop regulates Ras activity, in which ephrin ligand activates EphA2 receptor cell autonomously. Our results indicate that this is not likely. Examination of a large panel of breast cancer cell lines revealed that expression of EphA2 and its ligands is mutually exclusive. One explanation for this striking relationship appears to be that cells expressing ephrin ligand are of a different origin or lineage than those expressing EphA2. The expression of A-type ephrins (A1, A3, and A4) is restricted to cells that retain epithelial cell markers, and EphA2 is expressed in cells with mesenchyme characteristics (L. Timmerman, J.Y., J.W.G., and F.M., unpublished data). MDA-MB231 cells are the best characterized of this latter type: they display a fibroblast-like mor-

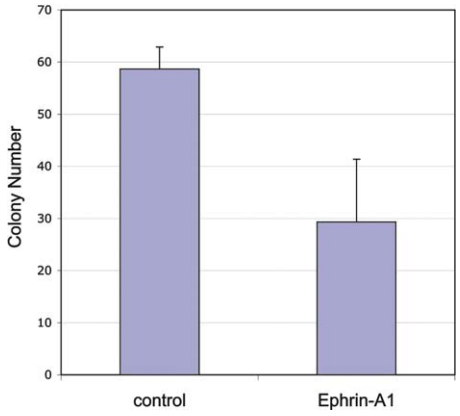

Figure 7. Ephrin-A1 inhibits transformation of NIH3T3 cells by v-ErbB in soft agar

Colony formation by v-ErbB was compared in the absence or presence of ephrin-A1 expression in NIH3T3 cells. Colonies were counted and graphed. The error bars represent an average of triplicates. The experiment was repeated twice.

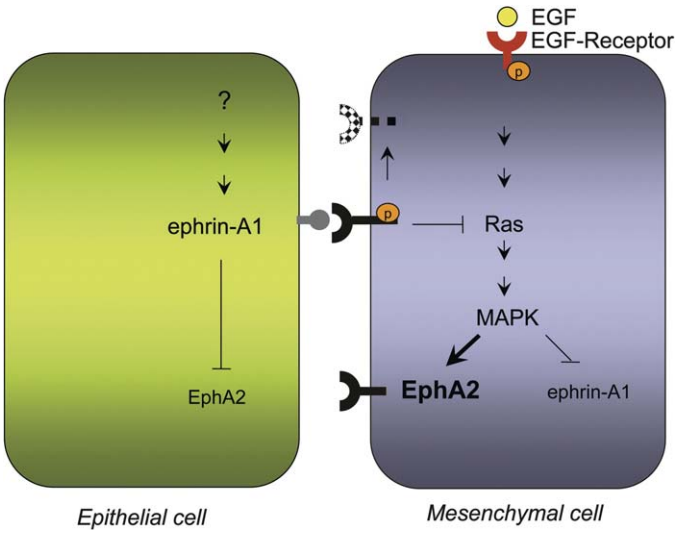

Figure 8. Model: A conditional feedback loop regulates Ras activity through EphA2

The MAPK pathway controls the expression of the EphA2 receptor and its ligand, ephrin-A1. Upon binding to ephrin-A1 (shown as a gray knob), the EphA2 receptor (shown in black) is phosphorylated, and the EGF-induced activation of the MAPK pathway is attenuated.

phology and grow as invasive colonies in matrigel, for example. They are an unusual example of a breast carcinoma cell that harbors mutant, activated *K-Ras* and *B-Raf* alleles. These cells have high constitutive levels of MAPK activity, and the highest level of EphA2 amongst the cells we examined. In these cells, elevated MAPK contributes to the suppression of ephrin expression: inhibition of the MAPK pathway induces ephrin expression while reducing EphA2 expression. The ability of the MAPK pathway to upregulate EphA2 while downregulating its ligand has a striking parallel: recently it was reported that β-catenin both activates transcription of *EphB* receptor and downregulates ephrin-B ligand, to determine cell fate in the intestinal epithelium (Batlle et al., 2002). It is possible that MAPK-EphA2 signaling plays a similar role in the organization of breast acini, but this remains to be proven. In breast cancer cells of the epithelial type, the expression of ephrin ligand contributes to EphA2 suppression, through a mechanism that consists, at least in part, of ligand-mediated receptor internalization (Figure 4E) and degradation (Sharfe et al., 2003; Walker-Daniels et al., 2002) but must also involve other regulatory processes that suppress EphA2 expression at the mRNA level. Interestingly, these cells have high levels of MAPK activity, but ephrin levels appear to be refractory to inhibition by MAPK. How these cells escape inhibition of ephrin by MAPK remains to be determined.

Finally, our results suggest that tumor cells whose phenotype depends on hyperactive Ras signaling may be suppressed by neighboring cells that present ephrin ligand on their cell surface, and that an escape from the negative effects of this interaction may be a necessary step in the development of such cancers.

Experimental procedures

Cell lines and cell culture

The NIH3T3:ΔRafAR and MCF10A:ΔRafER cell lines were received as gifts from Dr. Martin McMahon. The NIH3T3:v-ErbB cell line was a gift from Dr. Bill Weiss's laboratory. The breast cancer cell lines are part of the Breast Cancer SPORE project. Cells were grown at 5% CO_2 in DMEM or RPMI (as recommended by ATCC) containing 10% fetal bovine serum. Cells were serum starved for 24–32 hr prior to stimulation with hormone or EGF. Soluble ephrin-A1/Fc chimera and Fc were purchased from R&D Systems. EGF was purchased from Invitrogen. For coculture, ephrin-expressing T47D cells were harvested from a confluent plate, mixed with a subconfluent culture of EphA2 receptor-expressing BT549 cells, and incubated for 1 hr in a CO_2 incubator.

RNA preparation

Serum-starved NIH3T3:ΔRafAR cells were treated with 300 nM testosterone (Sigma) to stimulate the MAPK pathway. Total RNA was prepared using Qiagen's midi prep kit, and mRNA was prepared using Invitrogen's FastTrack kit.

cDNA clones and microarray analysis

A set of 15,552 mouse cDNA clones, consisting of 8,832 Incyte clones and 6,720 NIA clones obtained from the UCSF mouse consortium facility, were printed on glass slides (DeRisi et al., 1996). Typically, 1 μg mRNA or 10 μg total RNA were differentially labeled with Cy3 or Cy5 and used to hybridize the arrays for 16 hr and then washed (DeRisi et al., 1996). The arrays were scanned and analyzed using GenePix 3.0 software. The EphA2 and ephrin-A1 cDNA clones were obtained as gifts from Dr. Nigel Carter (Hunter Lab).

Northern blotting and quantitative RT-PCR

Ten micrograms of total RNA was resolved on a 1% formaldehyde gel. Nucleic acids were transferred to Hybond-N membrane (GE Healthcare) and probed with ^{32}P-labeled EphA2 cDNA. The membrane was autoradiographed using Hyper film from GE Healthcare. Quantitative RT-PCR was performed at the UCSF Cancer Center Genome core facility using an EphA2 primer set from Applied Biosciences.

Transfection

Cells were transfected with Lipofectamine 2000 per Invitrogen's transfection protocol and harvested 48 hr after transfection. To make stable cell lines, transfected cells were selected for drug resistance inherent to the plasmid construct. For siRNA transfections, 5 μg of siRNA smartpool from Dharmacon were transiently transfected into cells. To increase transfection efficiency, cells were retransfected 24 hr later and harvested 48 hr after the first transfection.

Protein gel electrophoresis and Western blotting

Cells were lysed in TNE buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA) containing 1% NP40, 1 mM DTT, protease inhibitor cocktail, and phosphatase inhibitor cocktails I and II from Sigma. Protein concentration was measured using BioRad's Lowry assay kit. Typically, 20 μg of total protein was resolved on 4%–20% Tris glycine SDS-PAGE gels (Invitrogen). Proteins were transferred to PVDF membranes (Millipore). Antibodies were used as recommended by the vendor. Primary antibodies used were as follows: polyclonal phospho-Erk and total Erk antibodies (Cell Signaling), monoclonal p21/Cip1 antibody (Becton Dickinson), monoclonal EphA2 and phospho-tyrosine 4G10 antibodies (Upstate), and anti-goat ephrin-A1 antibody (R&D Systems). Horseradish peroxidase (HRP)-conjugated secondary antibodies were as follows: anti-mouse (GE Healthcare), anti-rabbit (Cell Signaling), and anti-goat (Santa Cruz). Western blots were developed using enhanced chemiluminescence (ECL; GE Healthcare).

Immunofluorescence

EphA2-expressing cells were grown in 8-well chamber slides and treated with either 1 μg/ml soluble ephrin-A1/Fc or Fc for 0, 1, 3, or 7 hr. Cells were fixed with 4% paraformaldehyde and incubated with a monoclonal anti-EphA2 antibody for 1 hr followed by Alexa 488 secondary antibody (Molecular Probes). Cells were visualized by confocal microscopy.

Soft agar assay

NIH3T3 cells stably expressing either an empty vector or v-ErbB in the absence or presence of ephrin-A1 expression were mixed with 0.6% Sea Plaque agarose and plated in 6-well dishes (10^5 cells per well) containing a 1% Sea Plaque agarose cushion. Cells were allowed to grow at 37°C. Me-

dia were replenished once a week until colonies became visible. Colonies were stained overnight with 0.015% Neutral Red (Sigma #N-2889) and counted.

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