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Src Family Kinase Inhibitors Block Amphiregulin-Mediated Autocrine ErbB Signaling in Normal Human Keratinocytes

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Running title: SFKs regulate autocrine ErbB signalling in keratinocytes

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Non-standard abbreviations:

ADAM, a disintegrin and metalloproteinase

EGF, epidermal growth factor

ERK, extracellular regulated kinase

HRP, horseradish peroxidase

MMP, matrix metalloproteinase

NHK, normal human keratinocytes

PBS, phosphate-buffered saline

PBST, phosphate-buffered saline / 0.05% Tween 20

RTK, receptor tyrosine kinase

SFKI, Src family kinase inhibitor

TGF-α, transforming growth factor-α

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Abstract

c-Src potentiates proliferation, survival, and invasiveness in response to EGF in human mammary carcinoma cells. Tyrosine 845 of ErbB1 is phosphorylated by Src, and has been implicated in control of malignant behavior. While several lines of evidence also suggest important interactions of ErbB and Src family kinase signaling in normal epithelial cells, little is known about the mechanism of this interaction. Studying normal human keratinocytes (NHK), here we demonstrate strong expression of the Src family kinases Src, Yes, and Fyn; Src family kinase-dependent stimulation of Tyr 845 by EGF; and potent inhibition of NHK proliferation and migration by two Src family kinase inhibitors, PP1 and PD173952. EGF-stimulated ERK phosphorylation occurred at much lower concentrations of EGF than required to phosphorylate Tyr 845. Moreover, the effect of Src family kinase inhibitors on EGF-stimulated ERK phosphorylation was transient, prompting a search for other targets of Src family kinase action. By ELISA analysis, we found that three different Src family kinase inhibitors (PD173952, PP1, and SU6656) markedly inhibited elaboration of soluble amphiregulin by NHK. The ErbB inhibitor PD158780 and the MEK inhibitor U0126 also markedly inhibited NHK proliferation, migration, and amphiregulin production. Together, these observations demonstrate that one or more Src family kinases act upstream as well as downstream of ErbB1 to promote amphiregulin-dependent autocrine stimulation of NHK, and suggest that autocrine NHK proliferation is more dependent upon ERK activation than upon Tyr 845 phosphorylation.

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Introduction

The Src family kinases are a subclass of membrane-associated non-receptor tyrosine kinases (RTKs) involved in a variety of signal transduction processes, leading to a wide variety of cellular responses (Haskell et al., 2001; Thomas and Brugge, 1997). Of the nine known members of the Src family, six (Lyn, Lck, Hck, Blk, Fgr, and Yrk) are expressed primarily in hematopoietic cells, while three (c-Src, c-Yes, and Fyn) are expressed more ubiquitously. Attempts to ascertain the function of these three kinases by targeted ablation in mice have been complicated by functional redundancy (Lowell and Soriano, 1996). Most c-Src / Fyn and c-Src / c-Yes double mutants die in the perinatal period, whereas a substantial proportion of Fyn / c-Yes double mutants are viable but develop renal disease (Stein et al., 1994). Triple knockouts of c-Src, c-Yes, and Fyn manifest severe developmental defects, with lethality by embryonic day 9.5 (Klinghoffer et al., 1999). Together, these findings suggest that c-Src plays a particularly important role that can be complemented by Fyn and c-Yes.

While mutations in c-Src or other Src family kinases have only rarely been reported in human cancers, there is substantial evidence for overexpression of c-Src in a wide variety of human tumors, particularly those of the breast and GI tract (Summy and Gallick, 2003). In tumors expressing sufficient amounts of both ErbB1 (the epidermal growth factor [EGF] receptor) and c-Src, stimulation of ErbB1 with EGF has been shown to promote activation of c-Src (Summy and Gallick, 2003). Src family kinase activity has also been implicated in signaling downstream of ErbB1 via the ERK pathway (Biscardi et al., 1998; Olayioye et al., 2001). Stimulation of cells overexpressing ErbB1 and Src with EGF also results in Src family kinase-dependent phosphorylation of Tyr 845 of ErbB1 (Tice et al., 1999). While this residue is apparently not a direct target of ErbB1 autophosphorylation (Tice et al., 1999), Src family kinase-mediated phosphorylation of this residue is critical for EGF-dependent proliferation and

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tumor formation by these cells, via a pathway involving activation of Stat5 (Biscardi et al., 1999; Kloth et al., 2003).

Several lines of evidence suggest important interactions of ErbB and Src family kinase signalling in the skin. Overexpression of TGF-α produces a skin phenotype characterized by epidermal hyperplasia and hyperkeratosis within the first week after birth (Vassar and Fuchs, 1991). Animals overexpressing TGF-α in skin manifest increased c-Src kinase activity in the skin during phorbol ester-induced epidermal hyperplasia (Xian et al., 1997). Very similar hyperplastic skin phenotypes have been reported in mice expressing constitutively-active or wild-type forms of c-Src targeted to the interfollicular epidermis, and these animals produce significantly more papillomas and/or carcinomas than wild-type littermates (Matsumoto et al., 2002; Matsumoto et al., 2003). Activated Src has been localized to the edge of scrape wounds made in keratinocyte monolayers (Yamada et al., 2000). Several studies, including our own, strongly implicate ErbB1 in this response (Ellis et al., 2001; Stoll et al., 2003; Tokumaru et al., 2000). Thus, substantial available evidence suggests that a signaling pathway involving Src family kinases and ErbB1 may be of physiologic relevance to wounded and cancerous skin. However, to date it has been unclear whether these kinases are acting upon the same pathway(s), and if so, whether the site(s) of Src family kinase action reside upstream of ErbB1, downstream of ErbB1, or both (Pierce et al., 2001; Prenzel et al., 2001).

We chose to study the interactions of ErbB and Src family kinase signaling in normal human keratinocytes (NHK) because ErbB-Src family kinase interactions have not been extensively investigated in normal epithelial cells. This system is also attractive because ErbB1 exclusively controls the ERK pathway response to EGF in NHK due to absence of ErbB4, down-regulation of ErbB3, and the sequestration of ErbB2 in intracellular vesicles (Stoll et al., 2001). We have also shown that NHK are capable of high levels of autocrine signalling through ErbB1 to ERK (Kansra et al.,

2002; Stoll et al., 2002) via a pathway involving metalloproteinase-mediated release of amphiregulin from the cell surface (Kansra et al., 2004). Here we demonstrate strong expression of Src family kinases in NHK. We also characterize Src family kinase-dependent phosphorylation of Tyr 854 of ErbB1 as well as p80 and gp140, two abundant tyrosine kinase substrates recently shown to be expressed by NHK (Brown et al., 2004). We demonstrate transient inhibition of EGF-stimulated ERK phosphorylation by concentrations of Src family kinase inhibitors (SFKIs) capable of producing a profound and long-lasting inhibitory effect on the proliferation and migration of NHK. Finally, we implicate Src family kinases in the control of metalloproteinase-mediated release of amphiregulin in NHK. Taken together, our findings indicate that Src family kinase(s) act both upstream and downstream of ErbB1 in an autocrine signaling pathway that is strongly ERK dependent, leading to proliferation and migration of NHK.

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Materials and Methods

Reagents: Mouse monoclonal antibodies (mAb) directed against phosphotyrosine (4G10) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse mAb directed against human ErbB1 included Clone 13 from Transduction Laboratories (Lexington, KY) and Ab-15 from Labvision (Freemont, CA). Rabbit polyclonal antibodies (pAb) specific for ErbB1 phosphotyrosines 845 and 1148 were from Cell Signaling Technology (Beverly, MA) and BioSource International, Inc. (Camarillo, CA), respectively. mAb and pAb specific for total and phosphorylated ERK were from Cell Signaling Technologies. The anti-Src mAb 2-17 was a kind gift from Dr. Sarah Parsons, the anti-Yes mAb was Clone 1 from Transduction Laboratories, and the anti-Fyn rabbit pAb was sc-16 from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-BrdU mAb was from Sigma. PD173952 (Kraker et al., 2000; Prasad et al., 2002) was kindly provided by Drs. Alan Kraker and Wilbur Leopold, Pfizer Global Research and Development, Ann Arbor, MI. PP1 (Hanke et al., 1996) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). GM6001 (Holleran et al., 1997), PD158780, and SU6656 (Blake et al., 2000) (Rewcastle et al., 1998) were purchased from Calbiochem (San Diego, CA). Unless stated specifically, all other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture: A431 human epidermoid carcinoma cells (Giard et al., 1973) as well as MDA-MB-453, MDA-MB-468 and MCF-7 human breast carcinoma cells (Cailleau et al., 1978; McGrath et al., 1974) were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM (A431, MCF-7) or DMEM/F12 (1:1) (MDA-MB-453, MDA-MB-468), containing 10% fetal bovine serum (FBS) and antibiotics. NHK were obtained from sun-protected adult skin by trypsin flotation and propagated in modified MCDB 153 medium (M154, Cascade Biologics, Portland, OR) as described (Stoll et al., 2001), with the calcium concentration set at 0.1 mM.

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Keratinocyte growth assays: Clonal-density growth assays were performed as previously described (Klein et al., 1992), except that 1,000 cells were plated per 60 mm dish (50 cells / cm^2) in complete M154 medium. After 3 days, microcolonies consisting of 2-8 cells had formed. At this time, the medium was replaced with fresh complete M154 containing the inhibitors to be tested, or DMSO control, at a final DMSO concentration of 0.1%. After permitting the cultures to propagate for 8 additional days, colonies were visualized by crystal violet staining and photographed.

Autocrine growth assays were performed by seeding NHK at a density of 2,000 cells / cm^2 in 12-well plates in complete M154 medium. After overnight incubation in complete M154 medium, cells were switched to basal M154 medium containing 0, 1 or 10 ng/ml EGF along with various concentrations of PD173952, PP1, or the ErbB RTK inhibitor PD158780. After 5 additional days, growth was assessed using the MTT assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

To specifically assess DNA synthesis, BrdU incorporation assays were performed. NHK were plated on glass coverslips at 5,000 cells/cm2 and incubated for 24 h in complete M154 medium. The coverslips were then incubated in complete M154 medium containing 1:1,000 volume of DMSO (control) or the following inhibitors: PD173952 (0.2 and 1 μM), U0126 (10 μM), or PP1 (25 μM). 24 hr later, the cells were incubated with 50 μg/ml BrdU for 24 h, washed twice in phosphate-buffered saline (PBS), and fixed in 70% ethanol for 30 min at room temperature. Coverslips were then washed in 1X PBS and incubated with 4N HCl for 30 min, followed by 3 washes in 1X PBS and one wash in PBS containing 0.05% Tween 20 (PBST). Coverslips were then incubated with anti-BrdU mAb (used as ascites fluid at 12 μg/ml) in PBST for 1 hr at 37°C. After 3 further PBS washes, coverslips were incubated with 15 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 45 min at 37°C, then washed thrice in PBS, mounted in antifade mounting medium, and visualized under a Zeiss Axioskop microscope equipped for fluorescence. Digital imaging was achieved using a 2.2

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megapixel diode array camera (Optronics, Goleta, CA). To assess EGF-stimulated DNA synthesis, NHK were growth factor-depleted for 40 hr in basal M154 medium, then the same compounds were added for 1 hr followed by treatment with 1 ng/ml EGF or PBS control and 50 μg/ml BrdU for an additional 24 hr, prior to immunostaining for BrdU incorporation as described above.

Keratinocyte apoptosis assays: NHK were seeded at 5,000 cells / cm^2 and grown on glass coverslips in complete M154 medium until 70% confluent. Coverslips were then incubated with PD173952 (0.2 or 1 μM) for 24 or 96 hr, followed by washing in PBS and fixation for 30 min in freshly-prepared 4% paraformaldehyde. Coverslips were then washed with PBS, incubated with 0.5% Triton X-100 in 0.1 mM sodium citrate for 2 min on ice, rinsed in PBS, and then assayed for DNA fragmentation using a commercially-available *in situ* TUNEL assay kit (Roche Applied Science) according to the manufacturer's instructions.

Keratinocyte scratch wound migration assays: NHK were plated at 5,000 cells/cm^2 in 60 mm dishes and grown in complete M154 medium until they were approximately 90 % confluent. The dishes were scratched with blue pipette tips (1000 μl), washed two times with 1 x PBS and incubated in basal M154 medium at 37^{o}C / 5% CO2 in the presence or absence of EGF (10 ng/ml) with and without PP1 (5 or 25 μM), PD173952 (0.2 or 1 μM) or U0126 (2 or 10 μM). After 18-24 h, keratinocyte migration was assessed by phase contrast microscopy and documented by photography.

Cell stimulation and lysis: Prior to use in experiments, NHK were deprived of all growth factors by switching the medium to basal M154 medium for 1-2 days. After washing twice with Solution A [22.5 mM HEPES, 7.5 mM glucose, 2.25 mM KCl, 97.5 mM NaCl, 0.74 mM $Na_2HPO_4 \cdot 7\ H_2O$, pH 7.4], NHK were pretreated with inhibitors or DMSO control (1:1000 v:v) in fresh basal M154 for 15 min to 1 hr at 37^{o}C, and then were either left untreated or stimulated with EGF (1-100 ng/ml) for various times (5-120 min). After stimulation, NHK lysates were prepared by addition of non-ionic detergent

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lysis buffer [1% NP-40, 50 mM Tris pH 7.5, 5 mM EGTA, 120 mM NaCl, 20 mM □-glycerophosphate, 15 mM sodium pyrophosphate, 10 ug/ml leupeptin, 10 ug/ml aprotinin, 0.5 mM PMSF, 10 mM sodium orthovanadate, 50 mM sodium fluoride, 20% glycerol] on a rocker at 4oC for 5 min. 1 ml of lysis buffer was used for 100 mm dishes, and 0.5 ml of lysis buffer was used for 60 mm dishes. The resulting cell lysate was collected and clarified by spinning at 12,000 rpm for 5 min. Protein concentrations were estimated using the Bio-Rad DC Assay Kit (Bio-Rad, Richmond, CA). Tumor cell lines were harvested similarly, except that the lysis buffer was Laemmli gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol).

Western Blotting: Equal amounts of protein (10-40 μg) were electrophoretically separated on 4-20% Tris-glycine gels (Invitrogen, San Diego, CA) and transferred electrophoretically onto PVDF membranes (Invitrogen) according to the manufacturer's directions. Filters were rinsed using Dulbecco's phosphate-buffered saline (PBS) containing (0.1% Tween 20 (PBST), then incubated in blocking buffer (PBST containing 5% non-fat dry milk) with gentle rocking at room temperature for 30-60 min. Primary antibody incubations were done overnight at 4^{o}C in blocking buffer with gentle rocking at 1 μg/ml for anti-phosphotyrosine, anti-phospho-(845) ErbB1, and anti-phospho-(1148) ErbB1, and at 1:1,000 for anti-ERK or anti-phospho-ERK. After three rinses for 10 min each at room temperature with PBST, filters were incubated for 1 hr at room temperature with the appropriate secondary antibody in blocking buffer. Filters were again rinsed thrice in PBST, then detected on X-ray films using enhanced chemiluminescence as directed by the manufacturer (ECL, Amersham Biosciences, Piscataway, NJ).

ELISA assays for amphiregulin: NHK were seeded at 5,000 cells/cm^2 in 60 mm dishes and grown in complete M154 medium until they were approximately 40 % confluent. After rinsing twice with PBS, fresh basal M154 was added for an additional 24

hr, after which the conditioned medium was collected. After another two rinses in PBS, fresh basal M154 medium was added, containing PD173952 (0.1 to1 μM), PP1 (2.5 to 25 μM), GM6001 (40 μM), PD158780 (1 μM), U0126 (10 μM), or DMSO vehicle control (0.1% v/v). Two to twenty-four hours later, the conditioned media were collected, PMSF was added to 0.5 mM, and samples were stored at 4^{o}C for less than 10 days prior to assay. Amphiregulin was quantitated using an ELISA (R&D Systems) according to the manufacturer's instructions. Recombinant human amphiregulin (R & D Systems) was used as the standard, and the blank was M154 medium not exposed to cells. Samples with OD values > 2.0 were diluted 1:8. Results of single determinations from duplicate dishes were averaged, and these averages from independent experiments were used to determine standard errors of the mean. Statistical analyses are described together with the results.

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Results

Finding little information in the literature comparing the expression of different Src family kinases in NHK, we assessed the relative expression of c-Src, c-Yes, and Fyn in NHK by western blotting. As antibodies directed against different proteins cannot be assumed to be equally sensitive, we referenced our study to mammary carcinoma lines known to overexpress c-Src and ErbB1 relative to normal mammary epithelial cells (Biscardi et al., 1998). As shown in Fig. 1, NHK expressed c-Src at levels only slightly lower than those observed in mammary carcinoma cell lines and skin-derived A431 epidermoid carcinoma cells. c-Yes and Fyn and were also well-expressed by NHK. Indeed, Fyn was expressed at higher levels in NHK than in any of the carcinoma lines tested. High levels of ErbB1 expression were also observed in NHK, as previously reported (Stoll et al., 2001). From these results, we concluded that NHK express levels of Src family kinases and ErbB1 in the same range as malignant mammary epithelial cells previously found to overexpress ErbB1 and c-Src (Biscardi et al., 1998).

EGF-stimulated tyrosine phosphorylation of Tyr 845 of ErbB1 was found to be a distinctive feature of mammary carcinoma cells overexpressing c-Src and ErbB1 (Biscardi et al., 1998). While tyrosine phosphorylation of this residue is known to be Src family kinase-dependent in NHK (Wang et al., 2003) as well as breast carcinoma cells (Tice et al., 1999), its relationship to EGF stimulation has not been characterized in NHK. As shown in Fig. 2, NHK displayed dose-dependent phosphorylation of ErbB1 Tyr 845, with phosphorylation readily detectable at 10 and 100 ng/ml EGF, but not 1 ng/ml EGF. Interestingly, and in contrast to Tyr 845 phosphorylation, ERK phosphorylation was stimulated to near-maximal levels by 1 ng/ml EGF, as previously reported (Cai et al., 2002; Iordanov et al., 2002; Kansra et al., 2004). Indeed, we have recently demonstrated marked stimulation of ERK phosphorylation at EGF concentrations as low as 0.2 ng/ml in NHK (Kansra et al., 2004). Two different SFKIs, PD173952 and PP1, markedly and dose-dependently reduced EGF-dependent Tyr 845

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phosphorylation (Fig. 3); however, inhibition was incomplete. From these results, we conclude that high concentrations of EGF are required to elicit detectable Tyr 845 phosphorylation, and that kinases other than Src family kinases may participate in the EGF-dependent phosphorylation of Tyr 845 in NHK (see Discussion).

A more pronounced and complete inhibition of tyrosine phosphorylation was observed for two prominent bands of Mr 80 kDa and 140 kDa (Fig. 3A). These bands have been previously observed in NHK, where they have been designated p80 and gp140 (Brown et al., 2004). gp140 has recently been shown to be identical with CDCP1 (CUB domain-containing protein 1), a transmembrane cell-surface protein of uncertain function (Brown et al., 2004; Hooper et al., 2003). gp140 is heavily N-glycosylated, with a predicted molecular weight of 93 kDa based on amino acid sequence and a reduction in observed Mr on SDS-PAGE from 140 kDa to approximately 100 kDa after N-glycosidase F treatment (Hooper et al., 2003). Interestingly, p80 is a proteolytic fragment of gp140, which can be generated by trypsinization or plasmin cleavage of intact NHK (Brown et al., 2004). p80 and gp140 are single-pass transmembrane proteins, with the trypsin/plasmin cleavage site located in the extracellular domain and five of thirteen total tyrosine residues clustered in the cytoplasmic domain. Importantly, two laboratories have shown that tyrosine phosphorylation of p80 and gp140 is strongly Src family kinase-dependent (Brown et al., 2004; Hooper et al., 2003). Unlike phosphorylation of ErbB1 Tyr 845, tyrosine phosphorylation of p80 and gp140 was high even under conditions of growth factor deprivation, and was not markedly increased by EGF treatment (Figs. 2-4). Moreover, tyrosine phosphorylation of p80 and gp140 was not reduced by the ErbB RTK inhibitor PD158780 (data not shown). Regardless of the Src family kinase substrate examined, PD173952 was approximately 25 times more potent than PP1 on a molar basis (Fig. 3). Based on these findings, we conclude that p80 and gp140 are robust readouts for Src family kinase activity in intact NHK.

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However, tyrosine phosphorylation of these proteins does not appear to be under major control of ErbB1 signaling.

Of note, PD173952 and PP1 modestly decreased phosphorylation of ErbB1 Tyr 1148 in response to 10 or 100 ng/ml EGF (Fig. 3B). These observations raised the concern that these compounds might be directly inhibiting ErbB RTK activity. However, we found that PD173952 (Figs. 3A and 4A) and PP1 (Figs 3A and 4B) were ineffective in blocking the increase in ErbB1 Tyr 1148 phosphorylation stimulated by 1 ng/ml EGF. From these data, we conclude that PD173952 and PP1 are unlikely to be directly blocking the tyrosine kinase activity of ErbB1. (see Discussion).

Both PD173952 and PP1 inhibited EGF-stimulated ERK phosphorylation in a dose-dependent fashion. Again, PP1 was about 25-fold less potent than PD173952 in this regard. Interestingly, both inhibitors were more potent as inhibitors of ERK phosphorylation in response to 1 ng/ml EGF than in response to 10 or 100 ng/ml EGF (Fig. 3A). In particular, PP1 was not a very effective inhibitor of ERK phosphorylation in response to high concentrations of EGF, even after taking its lower intrinsic potency into consideration (Figs. 3A and B). In additional experiments (data not shown), high concentrations of PP1 (10-25 μM) inhibited EGF-stimulated ERK phosphorylation by less than 20% (as determined by visual estimation) in 8 of 8 experiments utilizing 10 ng/ml EGF for 10 min, and in 17 of 17 experiments utilizing 100 ng/ml EGF for 10 min. From these experiments, we conclude that while low concentrations of both SFKIs are very effective at inhibiting basal levels of ERK activation in NHK, they become progressively less effective as the concentration of EGF increases.

When 1 ng/ml EGF was used as the stimulus, the effects of both PD173952 and PP1 on ERK phosphorylation were much more pronounced at 5 min than at later times (Figs. 3A and 4). This transient inhibitory effect stands in contrast to the persistent inhibition of tyrosine phosphorylation observed for p80 and gp140 (indicated by asterisks in Figs. 3 and 4A). From these results, we conclude that Src family kinases

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exert a transient effect on EGF-stimulated ERK phosphorylation, which is most evident in response to low concentrations of EGF. Similar phenomena have been reported previously in other cell types (Olayioye et al., 2001) (see Discussion).

To explore the biological effects of Src family kinases in the context of ErbB signaling, we assessed the effects of PD173952 and PP1 on growth and migration of NHKs in the presence or absence of EGF. Treatment of sparsely-seeded (50 cells / cm^2) NHK cultures with either PP1 or PD173952 resulted in marked inhibition of colony growth (Fig. 5A). Again PD173952 was at least 25 times more potent than PP1, with near-complete inhibition of colony growth at a concentrations of 0.1 μM for PD173952 and 5 μM for PP1. Fig. 5B demonstrates that NHK colony growth was also inhibited in a dose-dependent fashion by the MEK inhibitor U0126, with substantial inhibition at 3 μM and complete inhibition at 30 μM.

The experiments shown in Fig. 5 were conducted using complete M154 medium, which contains 0.2 ng/ml EGF as well as insulin and bovine pituitary extract. To better assess the effects of SFKIs on NHK growth in the absence of EGF or other growth factors, we performed an autocrine growth assay as described in Materials and Methods. As long as microcolonies of 2-8 cells were allowed to establish themselves prior to removal of growth factors, NHK manifested robust autocrine growth, which could only be increased by approximately 50% by addition of EGF (Fig. 6). As assessed by a two-tailed t test with unequal variances, significant ($p < 0.005$) inhibition of autocrine NHK growth by PD173952 and PP1 was observed at all drug concentrations tested (Fig. 6). The existence of a dose response was confirmed by Spearman's correlation, with significance testing vs. 10^6 randomizations ($\rho = -0.938$, $p = 3 \times 10^{-6}$ for PD173952 and $\rho = -0.949$, $p = 2 \times 10^{-6}$ for PP1). The relative potencies of the two SFKIs were approximately the same as observed in assays of colony growth (Fig. 5A), Src family kinase-dependent tyrosine phosphorylation (Fig. 3), and ERK phosphorylation (Figs. 3

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and 4). A third SFKI, SU6656 (Blake et al., 2000), was also effective at inhibiting autocrine NHK growth at concentrations ≥ 2 μM (data not shown).

In order to better assess the interactions of Src family kinases and ErbB signaling driven by exogenous EGF in NHK, we added EGF (1 or 10 ng/ml) to some wells at the time the cells were switched from complete medium to basal medium. As assessed using Spearman's correlation, PD173952 and PP1 demonstrated significant ($p < 0.001$) inhibitory dose responses in the presence of 1 or 10 ng/ml EGF. Conversely, EGF exhibited a significant (Spearman's $p < 0.05$) growth-stimulatory effect at nearly all concentrations of either drug, with the only exception being 0.5 μM PD173952. Finally, the ErbB RTK inhibitor PD158780 (1 μM) significantly inhibited autocrine NHK growth ($p = 0.00009$ by two-sided t test with unequal variances), as well as growth in the presence of EGF ($p = 0.036$ for 1 ng/ml EGF and $p = 0.046$ for 10 ng/ml EGF). Together, these data suggest that the inhibitory actions of Src family kinases on NHK growth are mediated via an ErbB-driven autocrine loop, which can be "overdriven" to some extent by the addition of EGF.

To determine whether the growth-inhibitory effects of SFKIs on NHK reflect effects on proliferation, apoptosis, or both, NHK were subjected to BrdU incorporation and TUNEL assays. As shown in Fig. 7A, PD173952, PP1, and the MEK activation inhibitor U0126 markedly decreased BrdU incorporation by NHK cultured in complete medium over a 24 hr interval. PD173952 also inhibited EGF-stimulated BrdU incorporation in growth factor-depleted NHK, with marked inhibition even at 0.2 μM (data not shown). In contrast to their marked effects on proliferation and EGF-stimulated mitogenesis, PP1 and PD173952 had little effect on the number of apoptotic cells, as assessed by TUNEL assay (Fig. 7B). No TUNEL-positive cells were observed 1 day after treatment with either compound, and only scattered TUNEL-positive cells were seen after 4 days of treatment. Based on these results, we concluded that SFKIs exerted their effects primarily upon proliferation, rather than upon apoptosis.

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To assess the effects of SFKIs on wound-induced migration, subconfluent NHK monolayers were switched to growth factor-free basal M154 medium and scratched with a pipette tip. As shown in Fig. 8, NHK rapidly closed such wounds, with substantial closure observed by 20 h. Addition of EGF did little to speed closure. Both SFKIs were effective in inhibiting wound closure, with marked inhibition by as little as 0.2 μM PD173953 or 5 μM PP1. As previously demonstrated elsewhere (Stoll et al., 2003), U0126 also inhibited wound closure, demonstrating effectiveness at concentrations as low as 2 μM.

It was surprising that low doses of both SFKIs produced a long-lasting inhibition of NHK proliferation and migration (Figs. 5-8) and of basal levels of ERK phosphorylation (Fig. 4), despite producing only limited and transient inhibition of EGF-stimulated ERK phosphorylation at comparable doses (Fig. 4). Moreover, we observed that both PD173952 and PP1 totally inhibited the ERK phosphorylation observed in NHK under basal conditions; e.g., after 24-48 hr of growth factor deprivation without addition of exogenous EGF (Figs. 3B and 4). Based on our recent finding that amphiregulin is the major mediator of basal ERK phosphorylation in NHKs (Kansra et al., 2004), and on a report that Src family kinases are required for cleavage of the membrane-anchored form of HB-EGF in COS-7 cells (Pierce et al., 2001), we hypothesized that one or more Src family kinases might be necessary for expression and/or release of amphiregulin by NHKs. To test this hypothesis, we utilized a sensitive and specific ELISA to assess the effects of PD173952 and PP1 on elaboration of amphiregulin into NHK culture medium. As shown in Fig. 9A, left panel, PD173952 and PP1 markedly and significantly inhibited amphiregulin release over a 4-hr period, during which the concentration of amphiregulin in vehicle-treated control cells reached approximately 1 ng/ml. Significant inhibition was also observed for a third SFKI, SU6566, over an 8-hr period, during which the concentration of amphiregulin reached 2.5 ng/ml (Fig. 9A, right panel). The broad-spectrum metalloproteinase inhibitor

GM6001, the ErbB inhibitor PD158780, and the ERK activation inhibitor U0126 also markedly inhibited elaboration of amphiregulin over a 4-hr period (Fig. 9A, left panel). As shown in Fig. 9B, amphiregulin rose steadily under autocrine conditions (i.e., over a 24-hr period after medium change, and at least 48 hr after removal of exogenous growth factors), with significant inhibition of amphiregulin accumulation by PD173952 (0.5 μM) and PP1 (12.5 μM) at each time point. As shown in Fig. 9C, NHK displayed robust levels of ErbB1 tyrosine phosphorylation and ERK phosphorylation at 4 hr after medium change, which were markedly and significantly inhibited by both PD173952 and PP1. As expected, the ErbB inhibitor PD158780 also markedly inhibited autocrine ErbB1 tyrosine phosphorylation and ERK phosphorylation. GM6001 and U0126 also inhibited ERK phosphorylation, but surprisingly, ErbB1 tyrosine phosphorylation persisted to some extent after these treatments (see Discussion). Together, these findings demonstrate that Src family kinases play an important role in promoting amphiregulin release from NHK as part of an autocrine mechanism that also involves one or more MPs as well as activation of signaling from ErbB1 to ERK.

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Discussion

As reviewed in the Introduction, there is substantial evidence for an intimate connection between ErbB and Src family kinase signaling in normal and malignant epithelial cells, including keratinocytes. However, the mechanisms underlying this connection remain relatively unexplored, especially in normal epithelial cells. Normal keratinocytes undergo sequential alterations involving shape change, migration, proliferation, and survival in the context of wound healing. Each of these behaviors is known to be mediated by ErbB1 signaling in cultured NHK (Chen et al., 1993; Cook et al., 1991; Ellis et al., 2001; Jost et al., 2000; Pittelkow et al., 1993; Stoll et al., 1997; Stoll et al., 1998; Stoll et al., 2003; Tokumaru et al., 2000). Activation of Src family kinase signaling has also been shown to occur in the context of keratinocyte wounding (Yamada et al., 2000). Therefore, we hypothesized that one or more Src family kinases might be involved in ErbB1 signaling in normal, as well as malignant epithelial cells. This hypothesis predicts that Src family kinases should be well expressed in NHKs, that Src family kinases should signal through the same pathway(s) as ErbB1 in NHK, and that inhibition of ErbB or Src family kinase signaling should produce similar cellular phenotypes. Here we present evidence in support of each of these predictions, and provide evidence for Src family kinase regulation of autocrine ErbB signaling via control of amphiregulin release.

Fig. 1 demonstrates that c-Src, c-Yes, Fyn, and ErbB1 are well-expressed in NHK relative to various breast carcinoma cell lines, which overexpress these kinases relative to normal mammary epithelial cells (Biscardi et al., 1998). As we have previously reported (Stoll et al., 2001), ErbB1 is also well-expressed in NHK relative to mammary carcinoma cells. As expected, the express less ErbB1 than A431 cells, which are skin-derived epidermoid carcinoma cells (Giard et al., 1973) that have undergone ErbB1 gene amplification (Merlino et al., 1984). These findings demonstrate that despite their derivation from normal skin, NHK share with mammary carcinoma

cells and A431 carcinoma cells the property of high expression of both ErbB1 and multiple Src family kinases.

Fig. 2 demonstrates that Tyr 845 of ErbB1 is phosphorylated in an EGF-dependent manner in NHK. This residue is an autophosphorylation site in many other RTKs, whereas phosphorylation of Tyr 845 of ErbB1 is dependent on Src kinase activity (Tice et al., 1999). We found that high concentrations of EGF (10-100 ng/ml) were required for efficient phosphorylation of Tyr 845. Moreover, inhibition of EGF-stimulated Tyr 845 phosphorylation in NHK was much less complete than was observed for the recently-described (Brown et al., 2004) Src family kinase-dependent targets p80 and gp140 (Fig. 3). In contrast, Wang et al. have recently observed complete inhibition of Tyr 845 phosphorylation by 3 μM PP1 in skin carcinoma cells stimulated by engagement of β1 integrin by fibronectin (Wang et al., 2003). The β1 integrin-fibronectin interaction has been shown to promote ligand-independent, Src family kinase-dependent activation of ErbB1 (Moro et al., 2002). It is possible that Src family kinase-dependent phosphorylation of Tyr 845 is more robust in response to stimulation with high concentrations of EGF than in response to integrin ligation, and consequently harder to inhibit. Alternatively, Tyr845 may be a target of additional tyrosine kinases in NHK, which are activated in response to EGF exposure but not inhibited by SFKIs. Additional studies will be required to address this question.

It was concerning to us that both PD173952 and PP1 modestly reduced phosphorylation of Tyr 1148 in response to 10 or 100 ng/ml EGF (Fig. 3), because this residue is a known ErbB1 autophosphorylation site (Margolis et al., 1989). However, recent structural studies indicate that SFKIs should not reduce the intrinsic RTK activity of ErbB1 by virtue of their effects on Src family kinase-dependent Tyr 845 phosphorylation. Tyr845 is located within the activation loop of the catalytic domain of ErbB1, where its phosphorylation is thought to permit substrate binding in most RTKs (Biscardi et al., 2000; Huse and Kuriyan, 2002). However, ErbB1 is distinctive in that

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the activation loop adopts the conformation normally observed in phosphorylated and activated kinases, even when Tyr 845 is not phosphorylated (Burgess et al., 2003). Nevertheless, it remained possible that the SFKIs we have used could exert non-specific inhibition of ErbB1 RTK activity. PP1 is a well-established SFKI (Hanke et al., 1996) whose selectivity has recently been found to be superior to many others commonly used to probe signal transduction (Bain et al., 2003). PD173952 is a recently-developed compound with potent and selective activity against Src family kinases (Kraker et al., 2000; Prasad et al., 2002). Notably, the effects of these inhibitors on Tyr 1148 phosphorylation were less robust than their effects on Tyr 845 phosphorylation, and much less robust than their effects on p80 and gp140 phosphorylation (Fig. 3). Most importantly, SFKIs failed to inhibit Tyr 1148 phosphorylation when NHK were stimulated with 1 ng/ml EGF (Figs. 3A and 4). Taken together, these findings indicate that PD173952 and PP1 do not directly inhibit ErbB1 RTK activity in NHK in response to low ligand concentrations expected to be encountered in physiologic conditions (Plata-Salaman, 1991). However, it remains possible that these compounds may be acting by interfering with the ability of Src family kinases or other unidentified kinases to promote tyrosine phosphorylation of ErbB1 after stimulation of NHK with high concentrations of EGF.

As shown in Fig. 2 and elsewhere (Cai et al., 2002; Iordanov et al., 2002; Kansra et al., 2004), ERK phosphorylation in NHK is markedly increased by concentrations of EGF that are barely sufficient to increase tyrosine phosphorylation of ErbB1. We doubt that this is due to differential antibody sensitivity, as the anti-phosphotyrosine antibody we have used is sufficiently sensitive to detect basal tyrosine phosphorylation in Fig. 2, and yet only a limited increase in tyrosine phosphorylation is accompanied by a near-maximal increase in ERK phosphorylation. As shown in Fig. 4, the ERK phosphorylation response to 1 ng/ml EGF can be blocked by low concentrations of either PD173952 (0.2 μM) or PP1 (10 μM), at which these compounds markedly inhibit p80/gp140

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phosphorylation (Figs. 3 and 4) and can reasonably be assumed to be specific for Src family kinases (Bain et al., 2003; Hanke et al., 1996; Kraker et al., 2000; Prasad et al., 2002). Interestingly, ERK blockade in response to these SFKI concentrations was maximal after 5 min of EGF treatment, then was largely lost (Fig. 7). This result was not due to loss of inhibitory activity, as both SFKIs (particularly PD173952) produced a persistent inhibition of p80/gp140 tyrosine phosphorylation (Figs. 3A and 4). With increasing concentrations of EGF, both inhibitors became less effective (Fig. 3). Both phenomena--transient ERK pathway inhibition and diminished SFKI effectiveness against high concentrations of EGF--have been reported previously for mammary carcinoma cells (Olayioye et al., 2001). The mechanism of transient Src family kinase involvement in signal transduction from ErbB1 to ERK remains unresolved. While the timing of Src family kinase involvement correlates well with the kinetics of EGF-stimulated ErbB1 internalization in NHK (King et al., 1990), we have shown that most ErbB1 molecules remain on or near the cell surface after stimulation of NHK with 1 ng/ml EGF (Kansra et al., 2004). Therefore, we suspect that the observed effects of SFKI on signaling from ErbB1 to ERK in NHK will not be explained by receptor internalization.

When used at high concentrations (1 μM), PD173952 remained effective in blocking ERK phosphorylation in response to 10 and 100 ng/ml EGF (Fig. 3), and its effects against 1 ng/ml EGF were long-lasting, rather than transient (Fig. 4A). PD173952 also appeared to be a somewhat more effective inhibitor of p80/gp140 tyrosine phosphorylation than was PP1 (Figs. 3 and 4). PP1 is a pyrrolo[2,3*d*]pyrimidine, whereas PD173952 is a pyrido[2,3*d*]pyrimidine. While we have argued that PD173952 does not inhibit ErbB1 RTK activity, it remains possible that it may inhibit one or more additional kinases that are not targeted by PP1.

NHK displayed a remarkable capacity for Src family kinase-dependent autocrine proliferation (Figs. 5-7) and wound-induced migration (Fig. 8) (Turchi et al., 2002;

Yamada et al., 2000). The inhibitory effects of the ErbB RTK inhibitor PD158780 and the MEK kinase inhibitor U0126 on NHK proliferation and migration were very similar to those of the SFKIs, suggesting that Src family kinases, ErbB, and ERK signals might be acting along the same pathway. Indeed, the preferential effects of PP1, PD173952, PD158780, and U0126 on proliferation, as opposed to apoptosis (Fig. 7) are consistent with action all four compounds along the ERK pathway, which promotes cell-cycle progression via control of cyclin D1 transcription in attached cells (Danen and Yamada, 2001). As just discussed, low concentrations of both SFKIs had dramatic effects on the proliferation and migration of NHKs (Figs. 5-8), processes that occur over a time scale of hours to days. Moreover, blockade of basal (unstimulated) levels of ERK phosphorylation by both SFKIs was long-lasting (Fig. 9C), in contrast to the transient effects of these compounds on EGF (1 ng/ml)-stimulated ERK phosphorylation (Fig. 4). As each of these autocrine responses are strongly ErbB1-dependent (Chen et al., 1993; Klein et al., 1992; Stoll et al., 2003; Tokumaru et al., 2000) (Figs. 6 and 8), we hypothesized that this paradox might be explained by an inhibitory effect of SFKIs on the process of autocrine ligand production by NHK. Consistent with our hypothesis, we found that the elaboration of amphiregulin into the culture medium was markedly inhibited by both PD173952 and PP1 (Figs. 9A and B). This inhibition coincided with a strong inhibition of ERK phosphorylation (Fig. 9C). To our knowledge, this is the first demonstration of a role for Src family kinases in the regulation of autocrine (as opposed to G protein-coupled) ErbB signaling at the level of ligand release.

Amphiregulin release and autocrine ERK phosphorylation were also markedly inhibited by the ErbB RTK inhibitor PD158780, the MEK activation inhibitor U0126, and the metalloproteinase inhibitor GM6001 (Fig. 9). PD158780 and U0126 also exerted a strong inhibitory effect upon NHK proliferation (Figs. 5 and 6) and PD158780, U0126 and GM6001 markedly inhibited NHK migration (Fig. 7) (Stoll et al., 2003). These findings argue that each of these signalling components plays an important role in

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autocrine activation of ErbB signaling in NHK. By analogy with the proposed mechanism of ErbB1 transactivation via G-protein coupled receptors (Pierce et al., 2001; Prenzel et al., 2001), we would speculate that the major site of Src family kinase action may reside in control of the metalloproteinase(s) involved in cleavage of amphiregulin (Fig. 10). Given that U0126 also blocks amphiregulin release (Fig. 9), we would further speculate that amphiregulin production is an ERK-dependent process involving synthesis, transport, processing, and/or proteolytic cleavage of amphiregulin (Fig. 10). Indeed, ERK pathway activation has been implicated in metalloproteinase-mediated cleavage of pro-HB-EGF (Gechtman et al., 1999). We do not favor the idea that the major site of Src family kinase action relevant to amphiregulin production resides between ErbB1 and ERK, as the effects of SFKIs on EGF- stimulated ERK phosphorylation are transient (Fig. 4) at doses in which their effects on amphiregulin elaboration and autocrine ERK phosphorylation are long-lasting (Fig. 9). We also do not favor the concept that SFKIs inhibit ErbB1 RTK activity under autocrine conditions, as neither PD173952 nor PP1 inhibited phosphorylation of ErbB1 Tyr 1148 in response to low concentrations of EGF (Figs. 3 and 4). The pronounced inhibition of Tyr 1148 phosphorylation shown in Fig. 9C contrasts with the lack of inhibition seen in Figs. 3 and 4 because the former experiments allowed amphiregulin to accumulate over a 4-hr period, whereas the latter experiments only allowed amphiregulin to accumulate for 1 hr or less. We have previously shown that a 1-hr interval is insufficient to allow autocrine ErbB1-to-ERK signalling to become re-established after medium change (Kansra et al., 2004).

The persistence of ErbB1 tyrosine phosphorylation after 4 hr of treatment with GM6001 and U0126 (Fig. 9B) provides a challenge to the model shown in Fig. 10, in that interference anywhere within the autocrine cycle would have been expected to interrupt ErbB autophosphorylation. However, as discussed earlier, it is important to recall that ErbB1 can undergo ligand-independent tyrosine phosphorylation (Moro et al.,

2002; Moro et al., 1998). Moreover, pathway blockade for longer intervals might produce unexpected responses in the context of a positive feedback loop (Wiley et al., 2003) . Additional studies will be required to understand these unexpected findings.

As discussed in the Introduction, Tyr 845 phosphorylation plays a critical role in EGF-dependent mitogenic stimulation of fibroblasts and mammary carcinoma cell lines overexpressing ErbB1 and c-Src, via a pathway involving activation of Stat5 (Kloth et al., 2003; Maa et al., 1995; Tice et al., 1999). However, in those studies, EGF-dependent growth under anchorage-independent conditions required high concentrations of EGF (40 ng/ml) (Maa et al., 1995). Indeed, we have found that high concentrations of EGF (10 and 100 ng/ml) promote phosphorylation of Stat5 in NHK (Yong Li and J.T.E., unpublished observations). However, NHK grew very well in the absence of exogenous EGF, and growth was near-maximally stimulated by 1 ng/ml EGF (Fig. 6). Based on these findings, we expect that proliferation of NHK will lack the critical dependence upon Tyr 845 phosphorylation manifested by carcinoma cell lines. Additional studies will be required to address this question.

In summary, our studies indicate that Src family kinases play a complex yet crucial role in the process of autocrine ErbB signaling in NHK, resulting in autocrine proliferation and migration of NHK. It is possible that epithelial tumor cells of various origins may usurp various aspects of this normal mechanism via overexpression of ErbB1, c-Src, and/or other Src family kinases. Future studies should identify the specific Src family kinases involved in the production of autocrine ligands, and identify the point(s) at which they act in the autocrine ErbB1 activation cycle that we and others have described. Our work thus far suggests that NHK are an excellent model system for pursuit of this endeavor.

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Footnotes

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Legends for Figures

Figure 1. c-Src, c-Yes, and Fyn are expressed in NHK.

Cell lysates were prepared from NHK and the indicated tumor-derived cell lines as described in Materials and Methods. 25 μg of non-ionic detergent lysate were loaded for each of two different strains of NHK, whereas 40μg of Laemmli lysates were loaded for the remaining cell lines. Different lysis buffers were used because it was found that a strong band present in ionic detergent lysates of NHK co-migrated with c-Src and reduced its detectability. This band, presumably a keratin, was absent from all the tumor cell lines (data not shown). After electrophoretic separation, replicate blots were decorated with antibodies recognizing the proteins indicated to the right of the autoradiograms. Times indicate chemiluminescence exposure times. Total ERK is shown as an approximate control for equal loading of signaling components.

Figure 2. Dose-dependent phosphorylation of Tyr 845 of ErbB1 by EGF.

Two different strains of NHK were deprived of growth factors for 48 hr, then treated for 10 min with varying concentrations of EGF as indicated above the autoradiograms. Non-ionic detergent lysates (20 μg / lane) were subjected to SDS-PAGE. After blotting, replicate filters were decorated with antibodies recognizing the proteins indicated to the right of the autoradiograms.

Figure 3. SFKIs reduce EGF-induced ERK phosphorylation in NHK with little or no inhibition of ErbB1 autophosphorylation.

A. Comparison of stimulation with 1 ng/ml vs. 100 ng/ml EGF. Growth factor-depleted NHK were pre-treated with the indicated concentrations of inhibitors or DMSO control

for 15 min, followed by treatment with 1 or 100 ng/ml EGF or PBS control for an additional 15 min. Replicate western blots were then decorated with antibodies detecting the proteins indicated to the right of the autoradiograms (pY = total tyrosine phosphorylated proteins). The results shown are representative of two experiments in which 1 and 100 ng/ml EGF were tested in parallel. In the phosphotyrosine panel, the mobility of ErbB1 is indicated by the arrowhead, and the mobilities of gp140 and p80 are indicated by asterisks. Note the marked inhibition of ERK phosphorylation, and of tyrosine phosphorylation of 80 and 140 kDa bands, as a function of SFKI treatment. Also note inhibitory effects of SFKIs on ErbB1 Tyr 845 phosphorylation in response to 100 ng/ml EGF. Phosphorylation of Tyr 1148 (a known ErbB1 autophosphorylation site) is less markedly reduced by SFKI pretreatment, especially after stimulation with 1 ng/ml EGF (see text for details).

B. Comparison of stimulation with 10 ng/ml vs. 100 ng/ml EGF. Growth factor-depleted NHK were treated with the indicated concentrations of inhibitors or DMSO control (lanes 0) for 1 hour, followed by treatment with 10 or 100 ng/ml EGF or PBS control for 5 min (left panels) or 15 min (right panels). Replicate western blots were then decorated with antibodies detecting the proteins indicated between the autoradiograms. Arrowhead indicates mobility of ErbB1, asterisk indicates mobility of gp140 (p80 is not shown in this figure because the filter was cut horizontally to examine expression of another protein). Various exposure times are shown for different antibodies to ensure that the responses shown are in the linear response range; however, for each antibody, exposure times for the left- and right-hand panels were the same. Two different exposure times are shown for phospho-ERK in order to more clearly demonstrate the effect of SFKIs on basal (autocrine) ERK phosphorylation. The results shown are representative of 3 or more independent experiments. Note that compared to 1 ng/ml EGF treatment (Fig. 3A), inhibition of ERK phosphorylation in response to 10 or 100 ng/ml EGF requires higher concentrations of SFKI, and SFKI pretreatment reduces EGF-stimulated ErbB1 Tyr

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1148 autophosphorylation to a greater extent after stimulation with 10 or 100 ng/ml EGF than it does when 1 ng/ml EGF is used for stimulation. As was also seen in Fig. 3A, note the pronounced inhibition in gp140 tyrosine phosphorylation in response to SFKI pretreatment.

Figure 4. Transient inhibition of ERK phosphorylation and lack of inhibition of ErbB1 tyrosine phosphorylation by SFKIs after stimulation with low concentrations of EGF.

A. Effects of PD173952. Growth factor-depleted NHK were treated with the indicated concentrations of inhibitors or DMSO control for 1 hour, followed by treatment with 1 ng/ml EGF or PBS control for 5, 15, or 30 min. Replicate western blots were then decorated with antibodies specific for the molecules indicated to the right of the autoradiograms. Arrowhead indicates the mobility of ErbB1, asterisk indicates mobility of gp140. Note the lack of inhibition of EGF-stimulated ErbB Tyr 1148 phosphorylation in response to PD173952, despite marked and dose-dependent inhibition of gp140 tyrosine phosphorylation by this compound. The results shown are representative of 3 independent experiments.

B. Effects of PP1. Growth factor-depleted NHK were pretreated with the indicated concentrations of PP1 or DMSO control for 1 hour, followed by treatment with 1 ng/ml EGF or PBS control for the times shown above the autoradiograms. Replicate western blots were then decorated with antibodies specific for the molecules indicated to the right of the autoradiograms. Note the lack of inhibition of EGF-stimulated ErbB1 tyrosine 1148 phosphorylation, and the transient inhibition of ERK phosphorylation by PP1. While the maximum PP1 concentration shown in this figure is 10 μM, we found that even 25 μM PP1 did not persistently inhibit ERK phosphorylation in response to 1

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ng/ml of EGF (data not shown). The results shown are representative of three independent experiments.

Figure 5. Src family kinase and MEK inhibitors block growth of keratinocyte colonies.

NHK were seeded at a concentration of 1,000 cells per 60 mm dish, allowed to proliferate for 2 -3 days in complete M154 medium, then switched to complete M154 containing the concentrations of inhibitors given above the dishes. After an additional 9-12 days, colonies were fixed and stained with crystal violet. Data shown are representative of similar results observed in three experiments. A. Treatment with the SFKIs PD173952 or PP1. B. Treatment with the MEK inhibitor U0126.

Figure 6. Autocrine keratinocyte growth assay. NHK were plated in 12-well dishes at 2,000 cells/cm^2 in complete M154 medium. After overnight incubation, the medium was switched to basal M154 containing the indicated concentrations of EGF and inhibitors given at the bottom of the figure. All inhibitor concentrations are in μM. After 5 days, cell growth was estimated using the MTT assay. "Cont" indicates DMSO control (0.1% final concentration). PD158780 (1.0 μM) was included in these experiments to demonstrate the dependence of autocrine NHK growth on ErbB RTK activity. Each independent data point was the mean of OD562 readings obtained for 2 or 3 identically-treated wells. Error bars indicate standard deviations, n = 3 independent experiments for all conditions. For each concentration of EGF, significance testing was performed for each concentration of drug vs. the DMSO control at that EGF concentration using two-sided t tests with unequal variances. *** indicates $p < 0.0005$, * indicates $p < 0.05$. The significance of the overall dose response was further assessed for each concentration

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of EGF using Spearman's correlation against 10^6 randomizations of the observed data. In the absence of EGF, $\rho = -0.938$, $p = 3 \times 10^{-6}$ for PD173952 and $\rho = -0.949$, $p = 2 \times 10^{-6}$ for PP1. In the presence of 1 ng /ml EGF, $\rho = -0.775$, $p = 1.2 \times 10^{-3}$ for PD173952 and $\rho = -0.982$, $p = 1 \times 10^{-6}$ for PP1. In the presence of 10 ng/ml EGF, $\rho = -0.807$, $p = 5.9 \times 10^{-4}$ for PD173952 and $\rho = -0.982$, $p = 1 \times 10^{-6}$ for PP1.

Figure 7. SFKIs block NHK proliferation, with little effect on apoptosis.

A. Effects on proliferation: NHK were plated at 5,000 cells/cm^2 in complete M154 medium. After 24 hours, they were treated with 1μM PD173952, 25μM μM PP1, or 10 μM U0126. After an additional 24 hours, BrdU was added for a third period of 24 hours. Immunodetection of incorporated BrdU was then carried out as described in Materials and Methods. In the representative images shown, 19 of 24 cells displayed strong nuclear BrdU positivity in the DMSO control, as opposed to 3 of 14, 1 of 13, and 1 of 19 cells for PD173952, PP1, and U0126, respectively. The result shown is representative of 3 independent experiments.

B. Effects on apoptosis: NHK were grown to 70% confluence then treated for 1 or 4 days with the indicated inhibitors or DMSO control, followed by in situ TUNEL assay as described in Materials and Methods. Arrowheads indicate apoptotic cells. The result shown is representative of two independent experiments. As a positive control, fixed and permeabilized cells were treated with 0.5 mg/ml DNase I for 10 min at 20°C prior to TUNEL assay. All cells were positive (data not shown).

Figure 8. SFKIs block migration of NHK in a scratch-wounding assay.

NHK were grown to near-confluence, then scratched with a 1,000 μl pipette tip. After rinsing twice with PBS to remove cell debris, medium was changed to basal M154

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medium with or without 10 ng/ml EGF in the presence or absence of the indicated concentrations of PP1, PD 173952, PD158780, or U0126, or with DMSO vehicle as a control (1:1,000 v:v). After 20 hr, the wounds were photographed by phase contrast microscopy. Results are representative of three independent experiments. We have previously reported the inhibitory effect of U0126 upon scratch wound closure in NHK (Stoll et al., 2003).

Figure 9. SFKIs inhibit production of soluble amphiregulin and autocrine ERK activation.

A. Inhibitory dose responses. NHK were plated at 5,000 cells / cm^2 and grown to 40-50% confluence in complete M154 medium, followed by 24 hr in basal M154 medium. Cells were then washed twice in PBS and fresh basal M154 medium was added, together with the indicated concentrations of inhibitors. After 4 hr (PD173952 and PP1, left panel) or 8 hr (SU6656, right panel), conditioned medium was collected from each dish, PMSF was added to 0.5 mM, and media were held at 4^oC until amphiregulin content of the conditioned media was analyzed by ELISA. Error bars represent standard errors of the mean. The number of independent experiments per condition is indicated at the top of the graphs. Significance testing was performed using two-sided t tests with unequal variances. *** indicates $p < 0.0005$, ** indicates $p < 0.005$, * indicates $p < 0.05$. Significance of dose responses for each drug was further assessed using Spearman's correlation, with p values based on 10^6 randomizations of the data. For PD173952, $\rho = -0.881$, $p = 2 \times 10^{-6}$. For PP1, $\rho = -0.691$, $p = 1.3 \times 10^{-4}$. For SU6656, $\rho = -0.728$, $p = 6.6 \times 10^{-3}$.

B. Time course of amphiregulin accumulation and inhibition by SFKIs. NHK were plated and GF-depleted as described in Fig. 9A legend. The medium was then replaced with fresh basal M154 medium containing PD173952 (0.5 μM), PP1 (12.5 μM),

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or DMSO control for 2, 4, 8, or 24 hr followed by harvesting of CM and ELISA assay for amphiregulin as described in the Fig. 9A legend. "24 hr pre" denotes assay of aliquots of the basal 154 medium used to GF-deplete the NHK for the 24 hours prior to SFKI treatment. Error bars denote standard error of the mean; the number of independent experiments per condition is indicated at the top of the graphs. Significance testing was performed for each drug vs. DMSO control at each time point using two-sided t tests with unequal variances. *** indicates $p < 0.0005$, ** indicates $p < 0.005$, * indicates $p < 0.05$.

C. Inhibition of autocrine ERK phosphorylation. NHK were plated at 5,000 cells/cm2 in complete M154 medium, grown until 40% confluent, deprived of growth factors by incubation in basal M154 medium for 24 hours, washed twice in PBS, then placed in fresh M154 medium. After 4 hours, cells were lysed and subjected to Western blotting as described in Materials and Methods. Replicate blots were decorated with the antibodies indicated to the right of the autoradiographs. This result is representative of 2-3 independent experiments, and comes from one of the experiments used to generate the data shown in Fig. 9A.

Figure 10. Model for Src family kinase-dependent autocrine stimulation of NHK proliferation and migration by amphiregulin.

Not all ErbB1 tyrosine phosphorylation sites on ErbB1 are shown. Bold "X" indicates long-lasting blockade of amphiregulin release by SFKI, thin "X" indicates transient effect of SFKI on EGF-stimulated ERK phosphorylation. Evidence for involvement of calcium, Src, and protein kinase C in the action of G-protein coupled receptors has been reviewed by Prenzel et al. (Prenzel et al., 2001). This drawing is modified from Fig. 3 of that review.

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Figures

Please note: Figures 1-10 are submitted as separate electronic files.

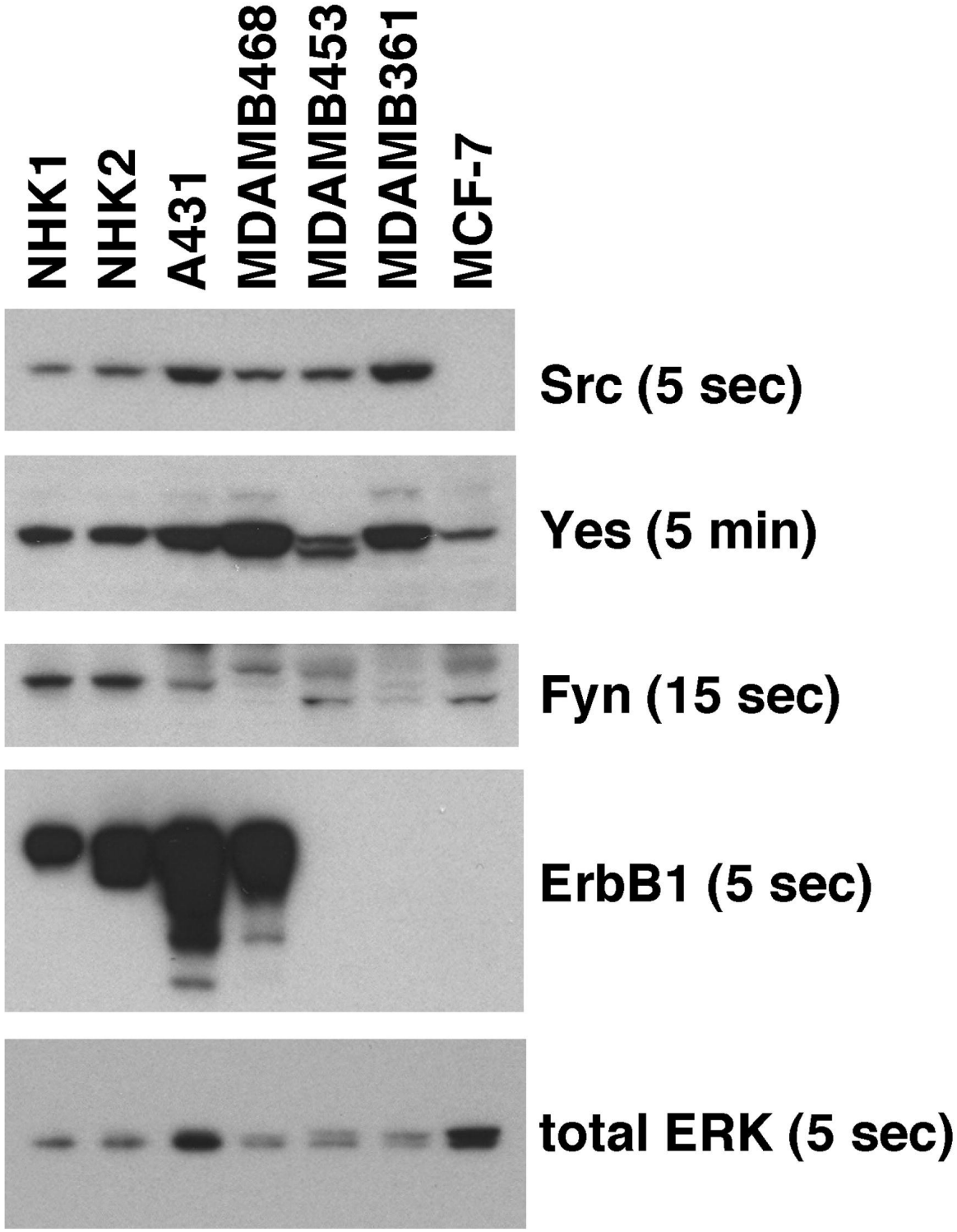

NHK1
NHK2
A431
MDAMB468
MDAMB453
MDAMB361
MCF-7
Src (5 sec)
Yes (5 min)
Fyn (15 sec)
ErbB1 (5 sec)
total ERK (5 sec)

Figure 1

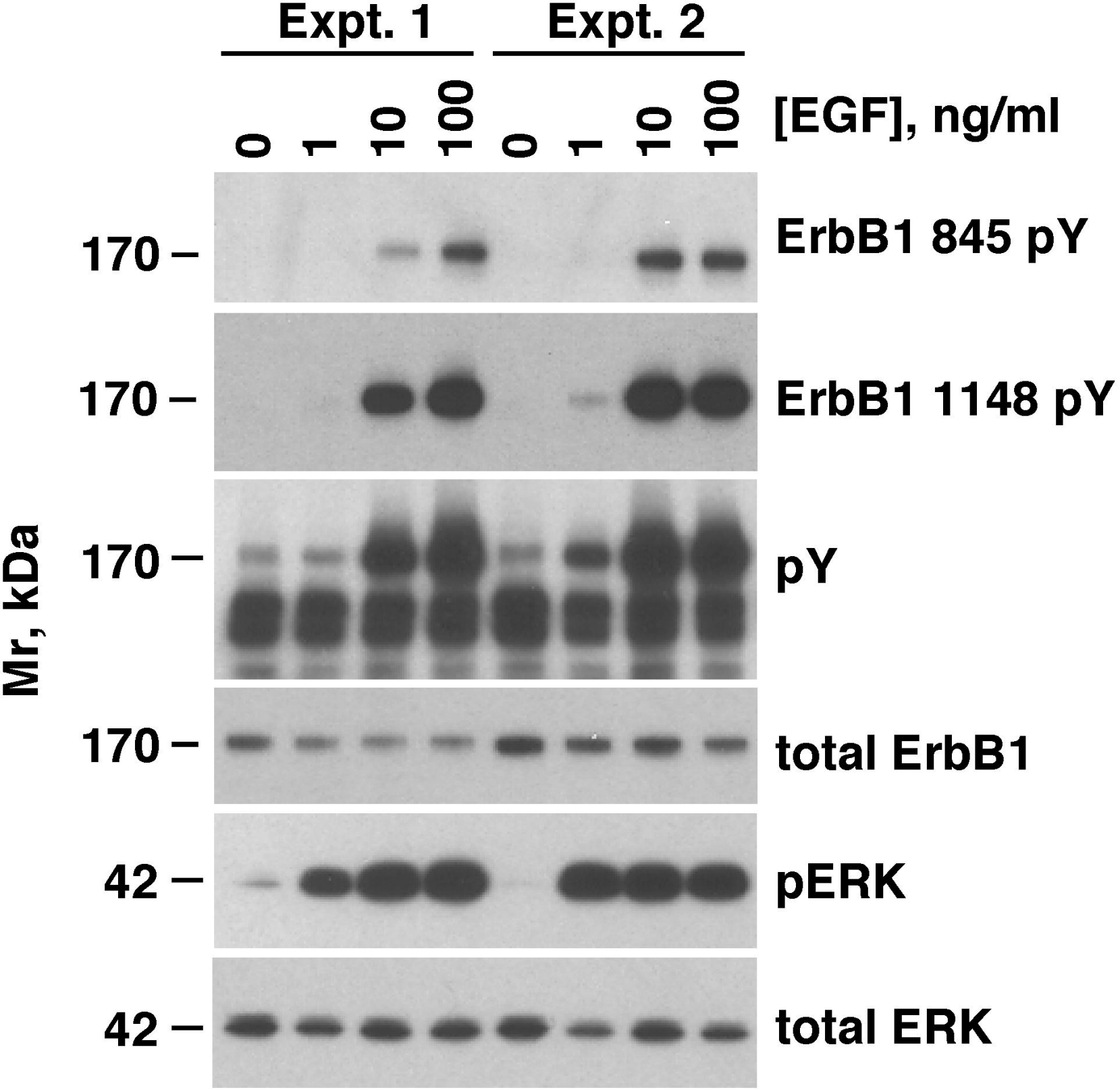

Expt. 1
Expt. 2
0
1
10
100
0
1
10
100
[EGF], ng/ml
170 –
ErbB1 845 pY
170 –
ErbB1 1148 pY
170 –
pY
170 –
total ErbB1
42 –
pERK
42 –
total ERK
Mr, kDa

Figure 2

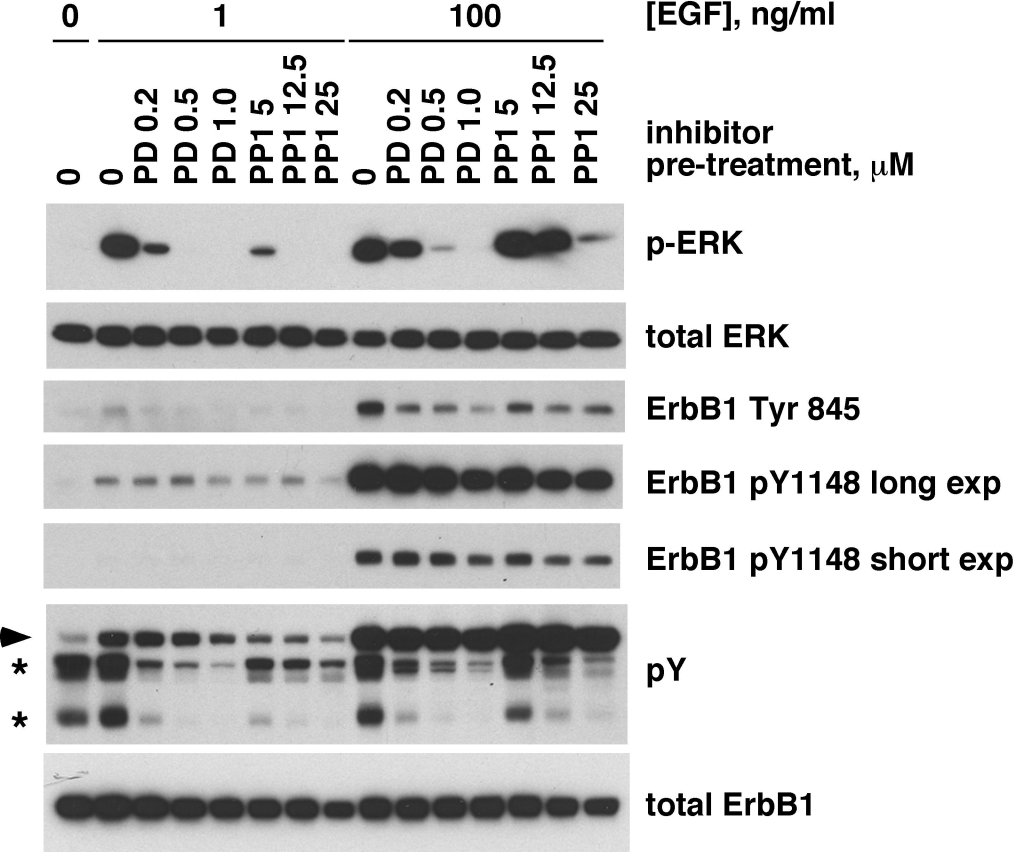

0
1
100
[EGF], ng/ml
0
0
PD 0.2
PD 0.5
PD 1.0
PP1 5
PP1 12.5
PP1 25
0
PD 0.2
PD 0.5
PD 1.0
PP1 5
PP1 12.5
PP1 25
inhibitor pre-treatment, μM
p-ERK
total ERK
ErbB1 Tyr 845
ErbB1 pY1148 long exp
ErbB1 pY1148 short exp
pY
total ErbB1

Figure 3A

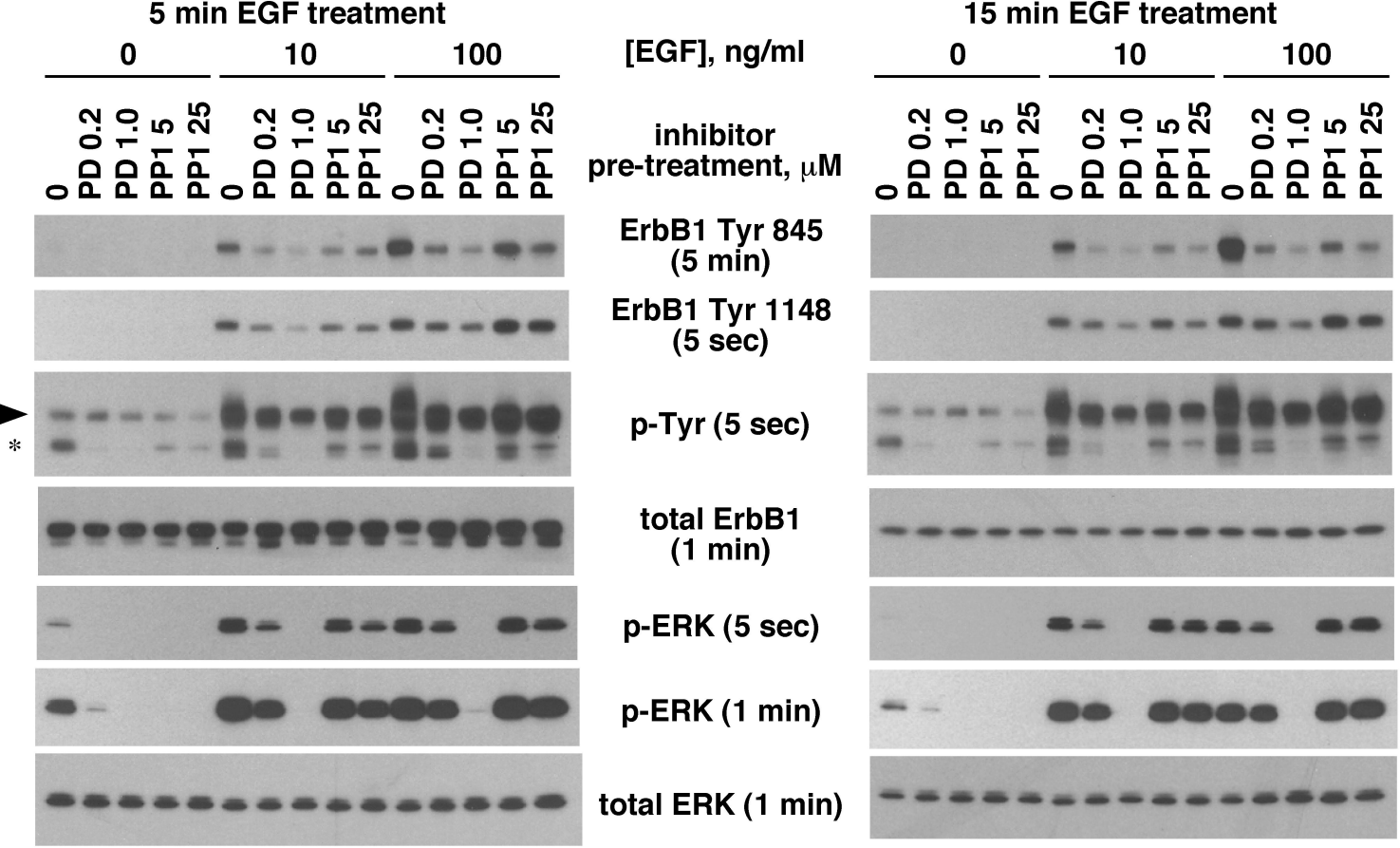

5 min EGF treatment
15 min EGF treatment
0
10
100
[EGF], ng/ml
0
PD 0.2
PD 1.0
PP1 5
PP1 25
inhibitor pre-treatment, μM
ErbB1 Tyr 845 (5 min)
ErbB1 Tyr 1148 (5 sec)
p-Tyr (5 sec)
*
total ErbB1 (1 min)
p-ERK (5 sec)
p-ERK (1 min)
total ERK (1 min)

Figure 3B

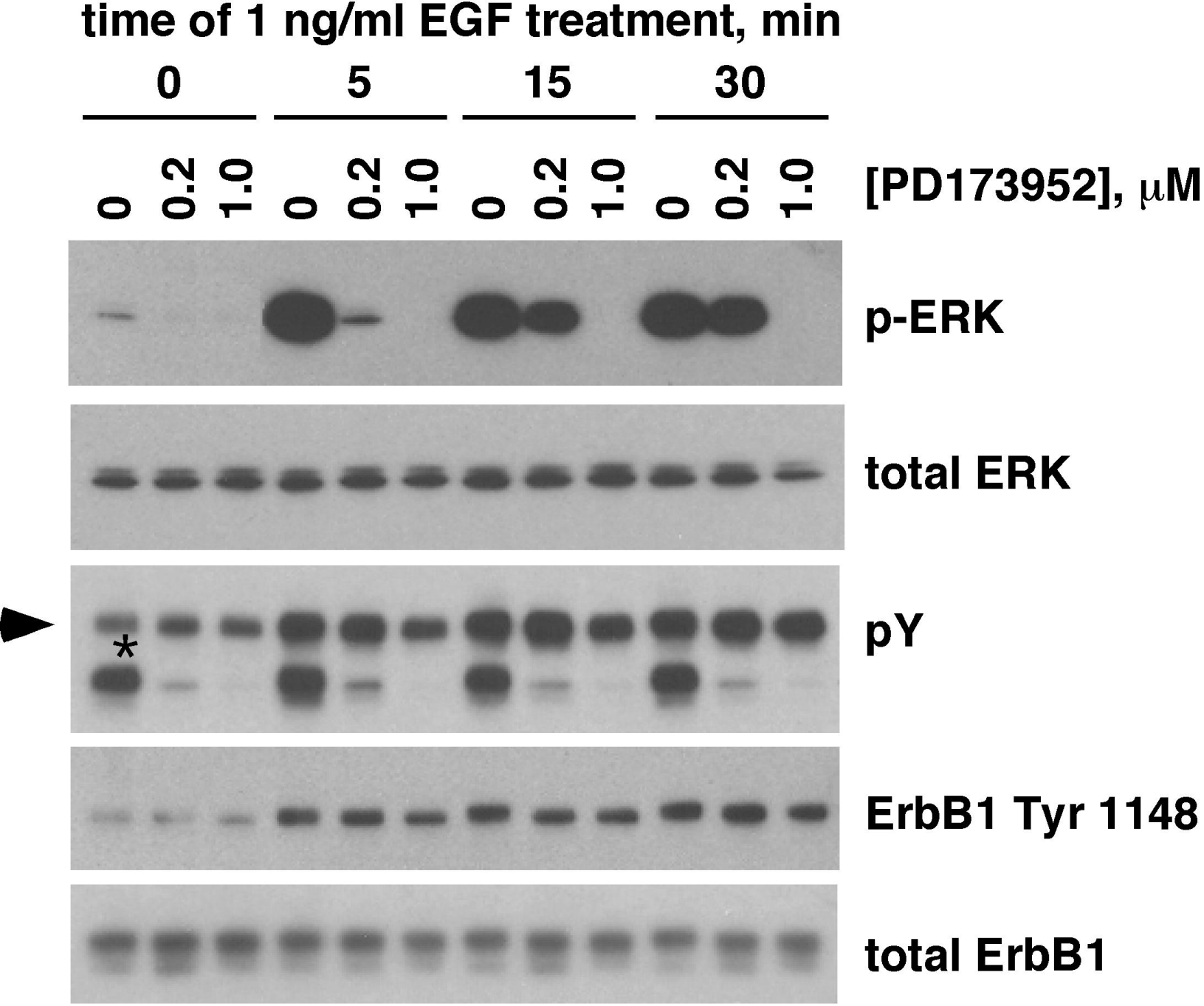

time of 1 ng/ml EGF treatment, min
0
5
15
30
0
0.2
1.0
[PD173952], μM
p-ERK
total ERK
pY
*
ErbB1 Tyr 1148
total ErbB1

Figure 4A

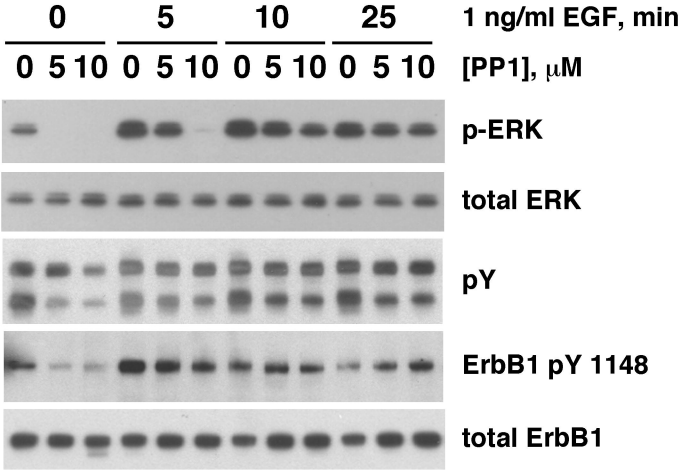
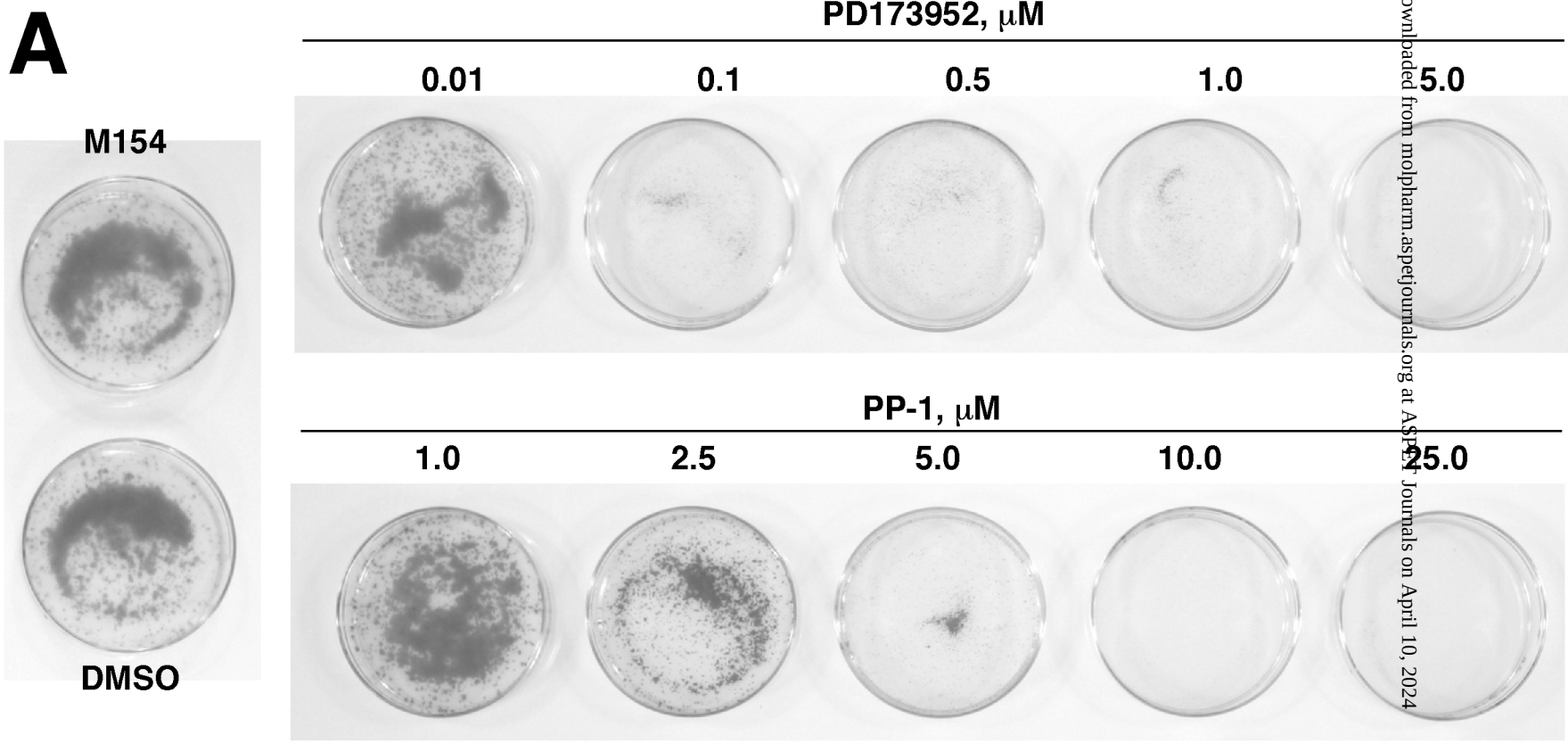

0
5
10
25
1 ng/ml EGF, min
0 5 10 0 5 10 0 5 10 0 5 10
[PP1], μM
p-ERK
total ERK
pY
ErbB1 pY 1148
total ErbB1

Figure 4B



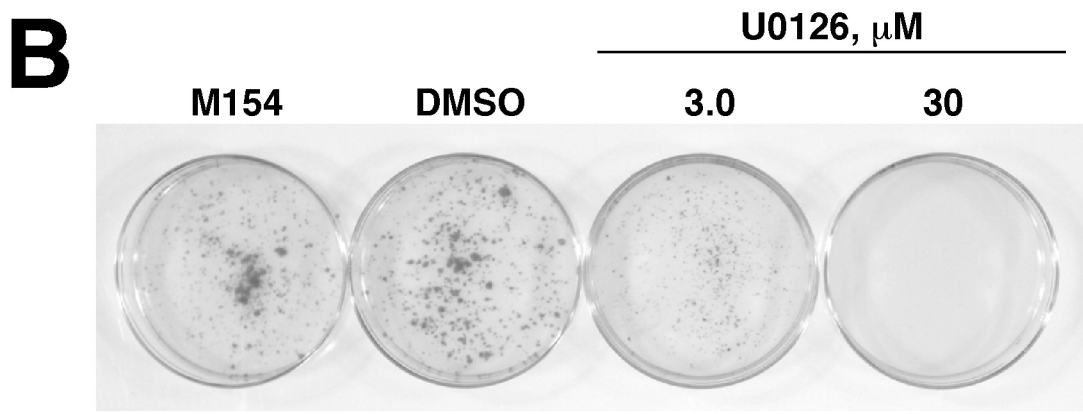


Figure 5

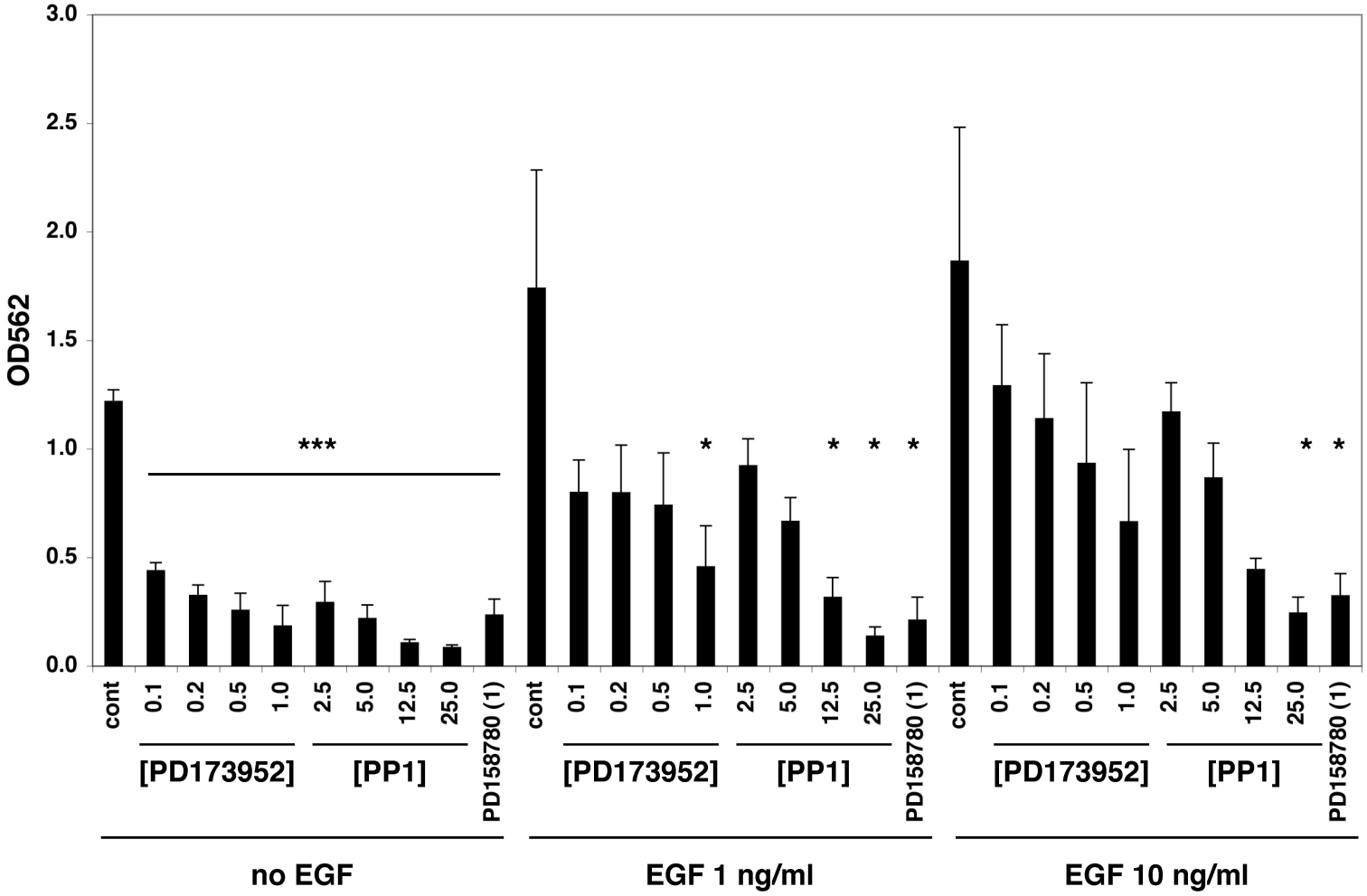

OD562
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
*
*
*
*
*
*
cont
0.1
0.2
0.5
1.0
2.5
5.0
12.5
25.0
PD158780 (1)
[PD173952]
[PP1]
no EGF
EGF 1 ng/ml
EGF 10 ng/ml

Figure 6

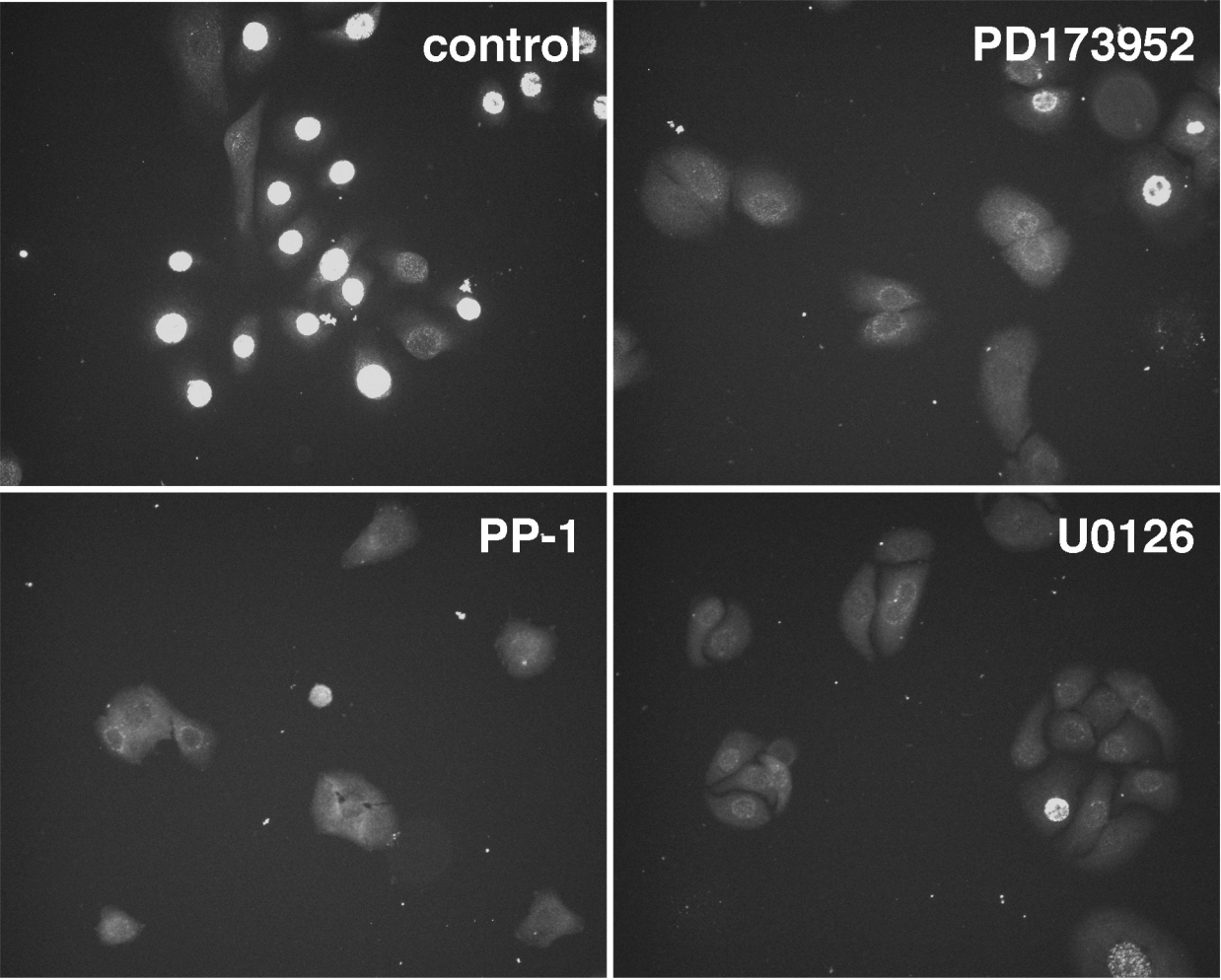


Figure 7A

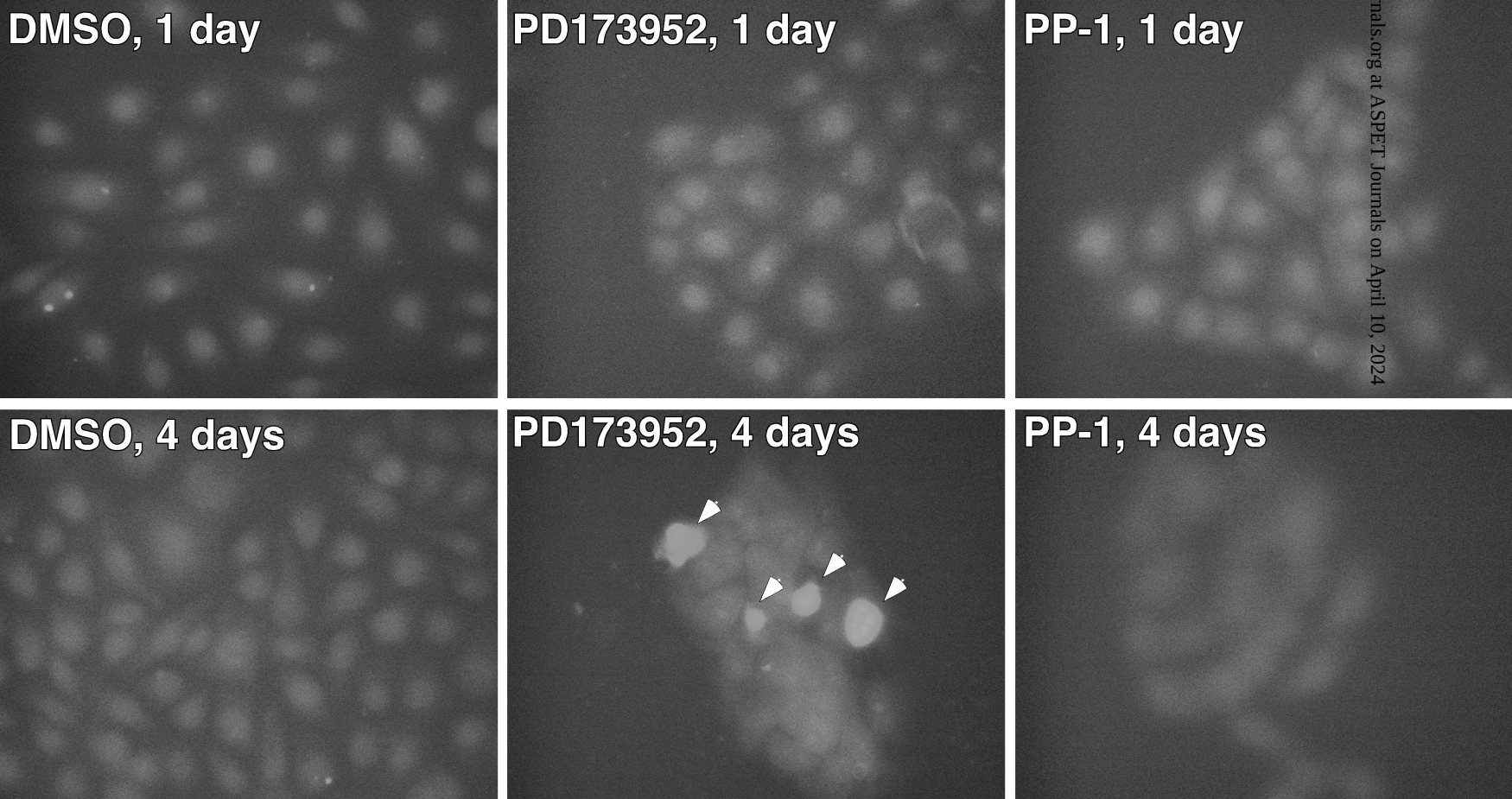


Figure 7B

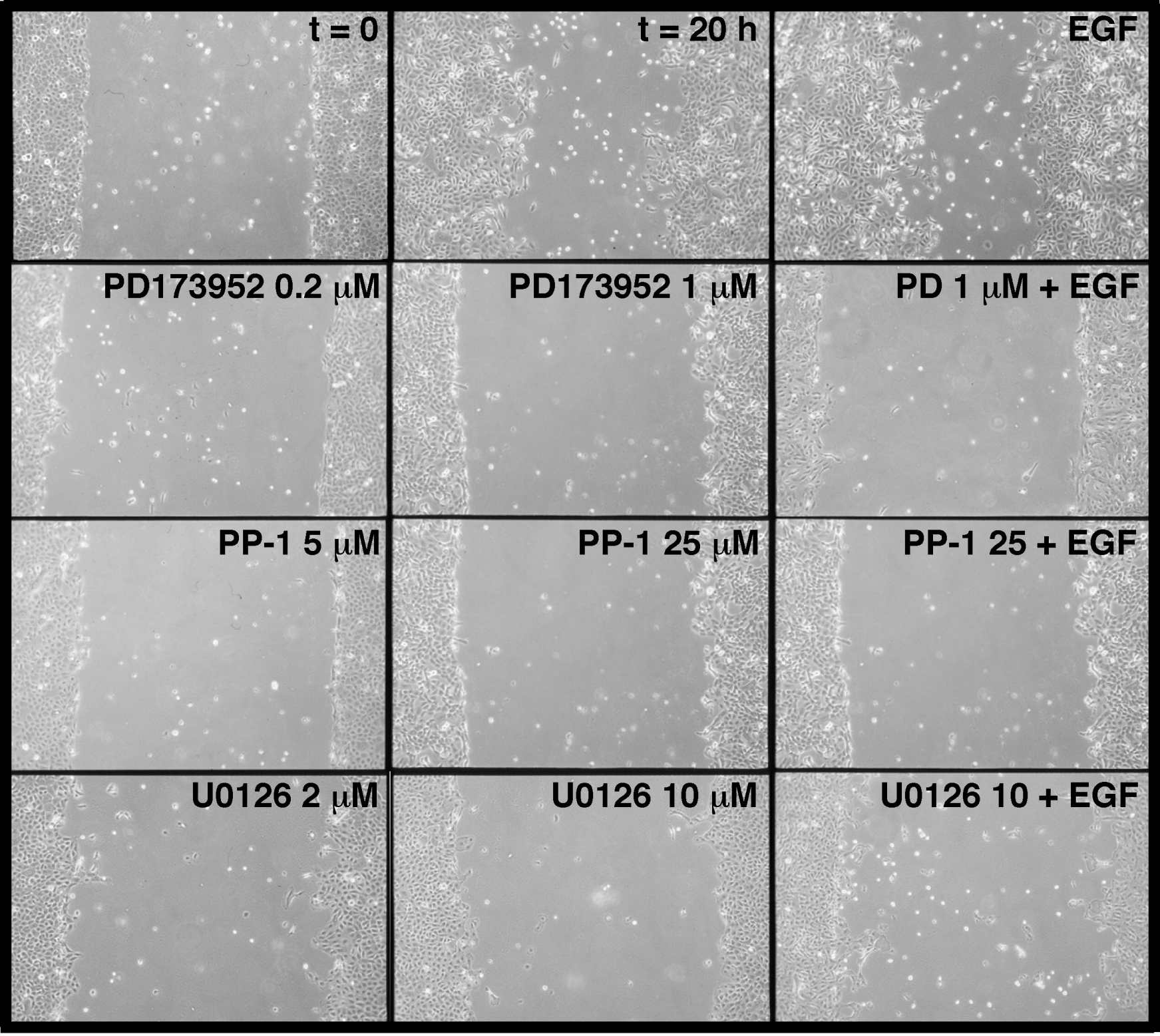
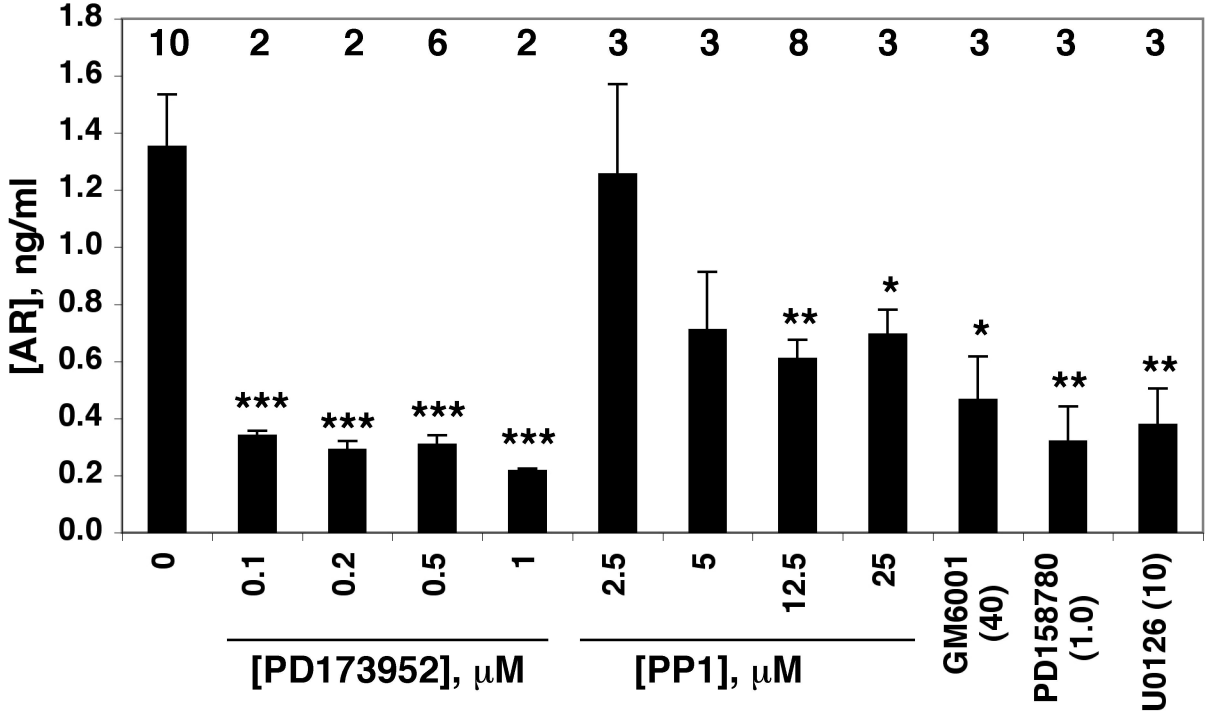

t = 0
t = 20 h
EGF
PD173952 0.2 μM
PD173952 1 μM
PD 1 μM + EGF
PP-1 5 μM
PP-1 25 μM
PP-1 25 + EGF
U0126 2 μM
U0126 10 μM
U0126 10 + EGF

Figure 8



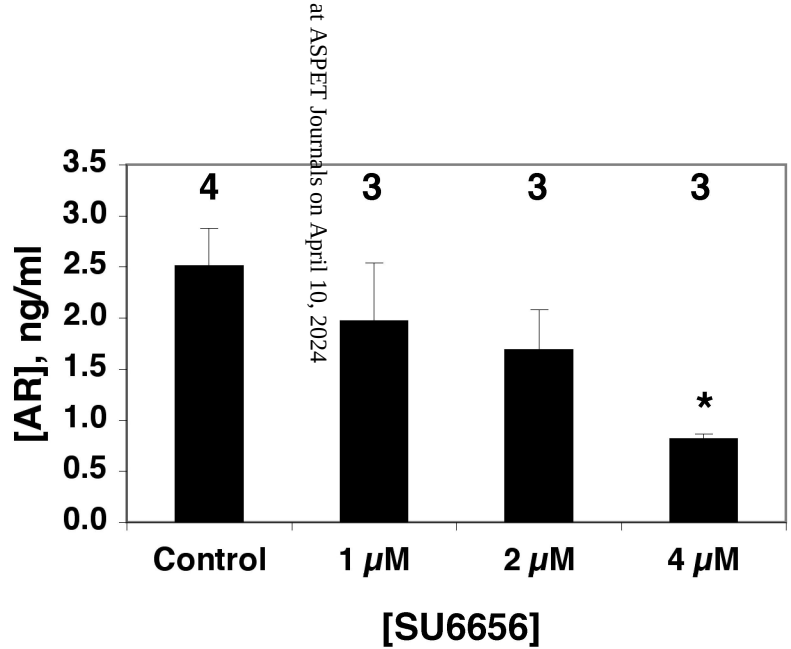


Figure 9A

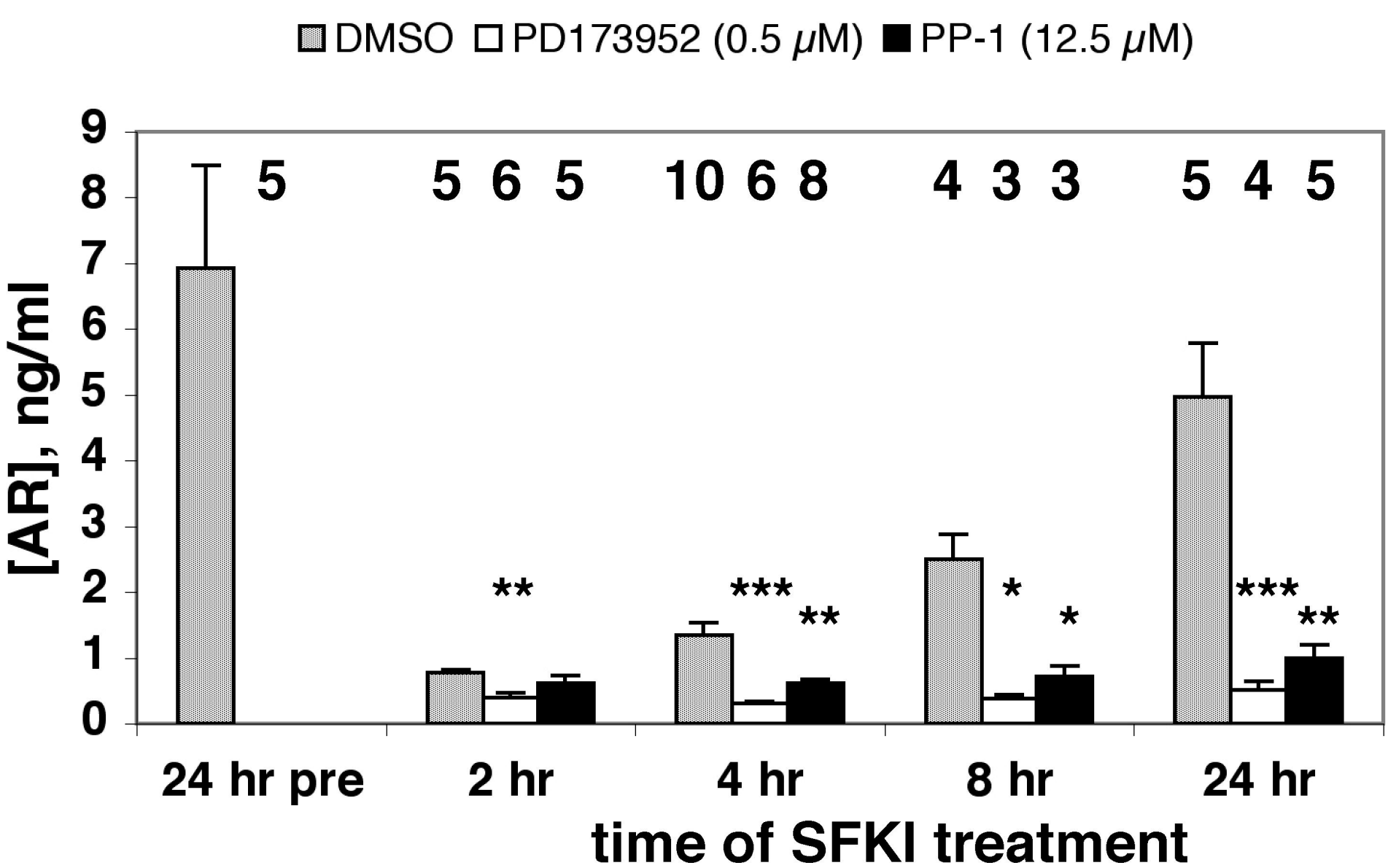

DMSO
PD173952 (0.5 μM)
PP-1 (12.5 μM)
[AR], ng/ml
0
1
2
3
4
5
6
7
8
9
5
5 6 5
10 6 8
4 3 3
5 4 5
**
***
**
*
*
***
**
24 hr pre
2 hr
4 hr
8 hr
24 hr
time of SFKI treatment

Figure 9B

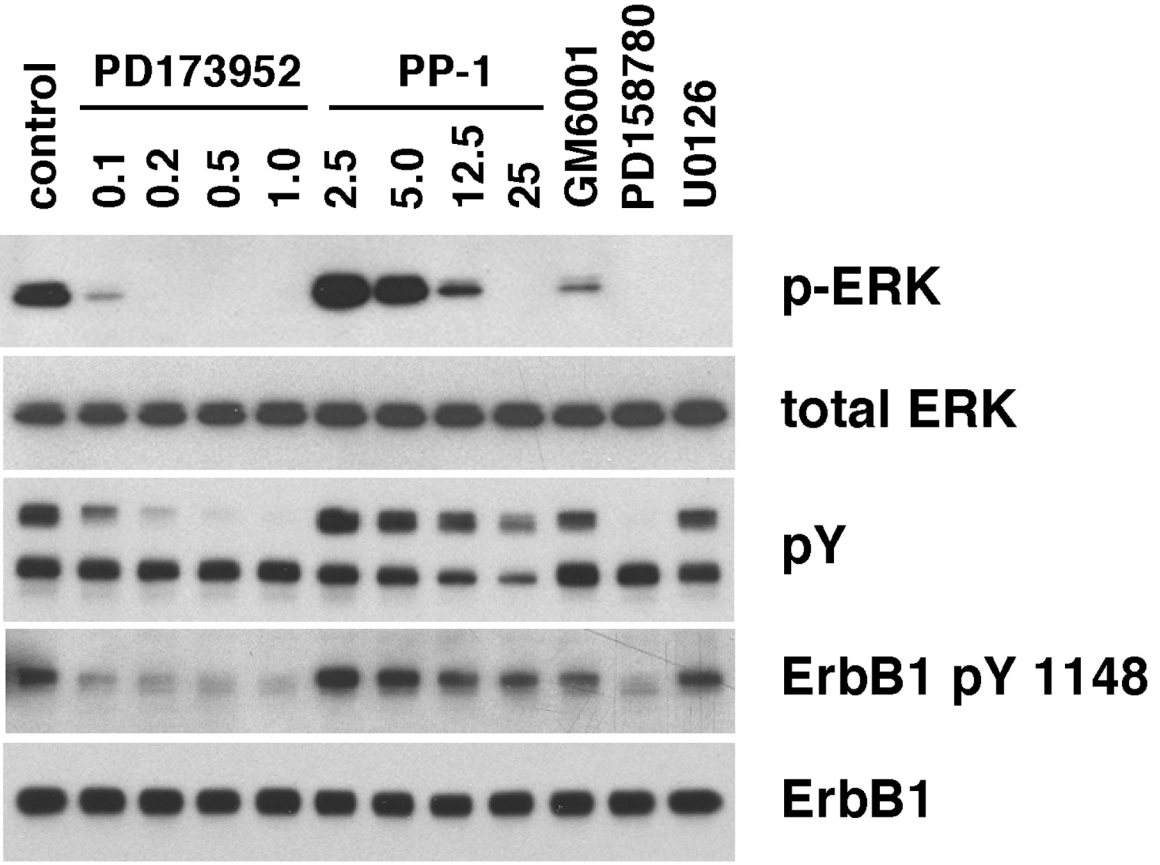

control
PD173952
0.1
0.2
0.5
1.0
PP-1
2.5
5.0
12.5
25
GM6001
PD158780
U0126
p-ERK
total ERK
pY
ErbB1 pY 1148
ErbB1

Figure 9C

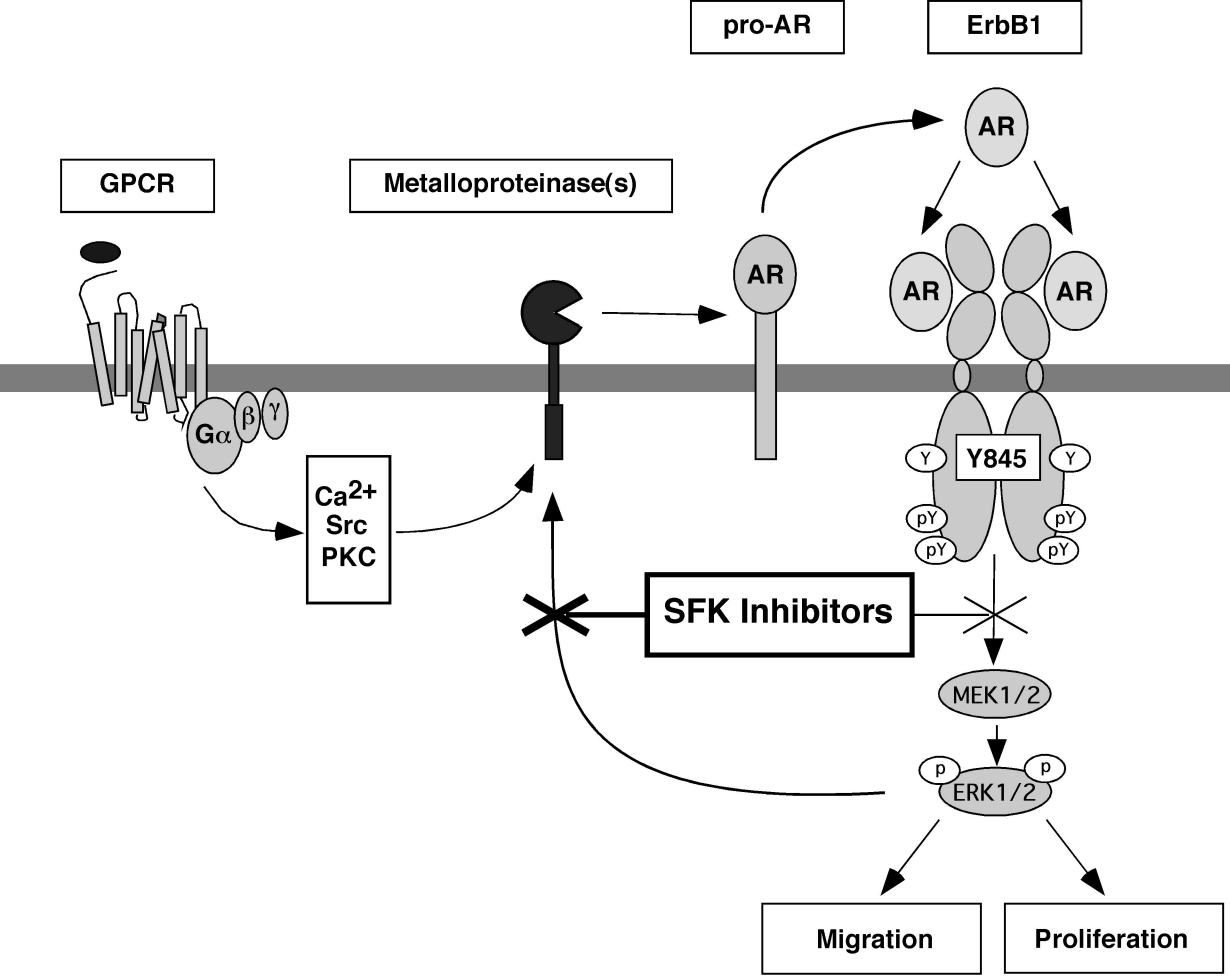

pro-AR
ErbB1
AR
GPCR
Metalloproteinase(s)
AR
AR
AR
Gα
β
γ
Y
Y845
Y
pY
pY
pY
pY
Ca2+
Src
PKC
SFK Inhibitors
MEK1/2
p
p
ERK1/2
Migration
Proliferation

Figure 10