Biological Role of Estrogen Receptor β in Salivary Gland Adenocarcinoma Cells

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Abstract

Purpose: This study is intended to investigate the biological role of estrogen receptor (ER) nongenomic signaling in salivary gland adenocarcinoma cells that predominantly express ERβ.

Experimental Design: Salivary gland adenocarcinoma cell lines HSG and HSY were used to study the effect of diarylpropionitrile and estrogen on the nongenomic signaling of ERβ, cytoskeletal remodeling, and cell motility.

Results: We found that diarylpropionitrile and estrogen triggered rapid activation of the extracellular signal-regulated kinase 1/2 (ERK), Src, and focal adhesion kinase signaling pathways. Estrogen stimulation also induced long cytoplasmic extensions, filopodia formation, and abnormal outgrowths in both HSG and HSY cells. We further observed that ligand-induced migration of these cells was blocked by the pure antiestrogen ICI 182780 and the mitogen-activated protein/ERK kinase inhibitor PD98059, indicating that estrogen-induced cell migration is mediated by the activation of ERβ nongenomic signaling.

Conclusion: These results clearly showed that ERβ nongenomic signaling is active in salivary gland cells and has a biological role in migration, presumably via the stimulation of ERK1/2. In future, the findings of this study might have clinical importance as several ERβ-selective agonists are currently being available, and these could potentially be used for therapeutic targeting of ERβ-positive salivary tumors.

The steroid hormone 17β-estradiol (E2) plays an important role in controlling the expression of genes involved in a wide variety of biological and neoplastic processes, including breast cancer progression (1). The biological effects of estrogen are mediated by its binding to the structurally and functionally distinct estrogen receptors ERα and ERβ, which mediate E2-targeted gene transcription, thus promoting the cell proliferation, differentiation, and migration of various cancer cells, including breast, endometrial, and lung cancer cells. In addition to the genomic actions of ERs, these receptors also have nongenomic functions, such as the activation of intracellular signal transduction pathways during the estrogen regulation of cell proliferation and migration.

In nongenomic mechanisms, E2-bound ERα activates various signal transduction pathways, including extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, Akt, c-Src, and focal adhesion kinase (FAK; refs. 2–4). Although the nongenomic potential of ERα has received much attention, the nongenomic potential of ERβ remains poorly understood. Two studies provided the first evidence of a functional ERβ in the gonadotropin-releasing hormone neurons and of the involvement of ERβ in nongenomic estrogen signaling in the brain. In the first, Chambliss et al. (5) found that a subpopulation of ERβ was localized to the endothelial cell plasma membrane; that overexpression of ERβ enhanced rapid eNOS stimulation by E2; and that the response to endogenous ER activation was inhibited by the ERβ-selective antagonist RR-tetrahydrochrysene. Abraham et al. (6) showed that estrogen acts rapidly and directly on the gonadotropin-releasing hormone neuronal phenotype, and that this action requires estrogen to pass through the cell membrane and interact with ERβ. Moro et al. (7) recently found that the nongenomic effects of estrogen are mediated through membrane-associated ERβ in enucleated platelet cells. In these cells, 17β-estradiol caused the rapid and transient tyrosine phosphorylation of Src and the formation of a membrane-associated, Src-dependent signaling complex, which includes ERβ, Src, Pyk2, and phosphatidylinositol 3-kinase. Collectively, these findings support a nongenomic signaling activation by estrogen coupled with ERβ in certain hormonally related tumors.

We recently investigated the expression of and the relationship between the novel hormonal receptor coactivator proline-rich, glutamic acid–rich, and leucine-rich protein-1 (PELP1) and ERβ in salivary duct carcinomas (8). In tumor cells, staining for PELP1 was predominantly cytoplasmic, whereas staining for ERβ was nuclear and occasionally cytoplasmic. Our results revealed that ERβ and PELP1 were coexpressed in most salivary duct carcinomas and may play a pathobiological role

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in these tumors (8). There was no detectable expression of ERα in any of the normal or salivary duct carcinoma specimens tested. PELP1 plays a role in genomic functions of ER via histone interactions (9). However, recent studies have shown that PELP1 in cytoplasm could play a crucial role in modulating nongenomic signaling by using molecular mechanisms that remain poorly understood. Moreover, because PELP1 is predominantly localized in the cytoplasm in a substantial proportion of human breast (10) and endometrial cancers (11), PELP1-induced nongenomic signaling is expected to have physiologic implications in tumorigenesis.

Recently, we showed that both estrogen and the selective ER modulator tamoxifen induce rapid activation of MAPK, Src, and FAK, leading to the formation of lamellipodia and actin spikes in human endometrial adenocarcinoma cell lines Hec 1A and Hec 1B (12). These findings are clear evidences that cell migration is regulated by the nongenomic signaling of E2.

Because ERβ is the predominantly expressed form of ER in salivary gland tumors and given that PELP1 is predominantly cytoplasmic in salivary gland tumors, we hypothesized that ligand-induced nongenomic signaling occurs through ERβ in salivary gland carcinoma cells. To better understand the cellular functions of ERβ in salivary gland carcinoma and to determine its role in nongenomic signaling, we analyzed the effect of diarylpropionitrile and estrogen on rapid nongenomic signaling of ERβ and its involvement in the regulation of cytoskeletal changes and migration of salivary gland carcinoma cells.

Materials and Methods

Cell cultures and reagents. The salivary gland adenocarcinoma cell lines HSG and HSY were generously provided by Dr. Fredrick Kay (NIH, Bethesda, MD). Breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in DMEM-F12 (1:1) supplemented with 10% fetal bovine serum. Estrogen was purchased from Sigma-Aldrich (St. Louis, MO). The ERβ-selective agonist diarylpropionitrile and the ER antagonist ICI 182780 were purchased from TOCRIS (Ellisville, MO). The ER ligand estren (4-estren-3α, 17β-diol) was purchased from Steraloids (Newport, RI). The MAP/ERK kinase inhibitor PD98059 was purchased from Promega (Madison, WI).

We used the following antibodies: ERα (Chemicon International, Temecula, CA); ERβ (Oncogene Research Products, San Diego, CA); phospho-p42/p44 ERK/MAPK, phospho-Src Tyr^{416}, Src (Cell Signaling, Beverly, MA); ERK1, ERK2, FAK (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-FAK Tyr^{397} (BioSource International, Camarillo, CA).

Cell extracts and immunoblotting. For cell extract preparation, cells were grown in 1% DCC medium for 48 hours and treated with either diarylpropionitrile (10 nmol/L), estren (10 nmol/L), or E2 (10 nmol/L). When indicated, ICI 182,780 (100 nmol/L) or PD98059 (10 μmol/L) were added 1 hour before the ligand treatment. Cells were washed twice with PBS and then lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1 mmol/L sodium vanadate] for 10 minutes on ice. The lysates were centrifuged in an Eppendorf centrifuge, at 4°C for 10 minutes. Cell lysates containing an equal amount of protein (~200 μg) were then resolved on an SDS-polyacrylamide gel (8% acrylamide), transferred to a nitrocellulose membrane, probed with the appropriate antibodies, and developed using either the enhanced chemiluminescence or the alkaline phosphatase–based color reaction method.

Immunofluorescent labeling and confocal microscopy. The cellular localization of proteins was determined by indirect immunofluorescence. Briefly, HSG or HSY cells were grown on sterile glass coverslips, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 5% normal goat serum-PBS. Cells were incubated with primary antibodies, washed thrice in PBS, and then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with Alexa 546 (red) or Alexa 488 (green) from Molecular Probes (Eugene, OR). The DNA dye Topro-3 (Molecular Probes) was used as nuclear stain (blue). Microscopic analyses were done using an Olympus FV300 laser scanning confocal microscope in accordance with established methods, using sequential laser excitation to minimize fluorescence emission bleed-through. Each image was obtained at the same cellular level and magnification.

Migration and wound-healing assays. To measure cell migration potential, HSG and HSY cells were serum starved (0% DCC) in phenol red–free medium for 48 hours. Cells were trypsinized for collection, washed in PBS, then resuspended in phenol red–free medium in the presence of 0.1% bovine serum albumin, and loaded on the upper well of an uncoated Boyden chamber at a concentration of 10,000 per well. E2 (10 nmol/L) was diluted in the cell medium before cell plating, as described for the individual experiments. The lower side of the separating filter was filled with a conditioned medium of NIH-3T3 fibroblasts grown in DMEM/F12 medium with 0.1% bovine serum albumin. We counted the cells that successfully migrated through the filter. Experiments were done in triplicate.

We also assessed the cell migration potential by using an established wound- healing assay as previously described (12). Briefly, HSG or HSY cells were plated in 60-mm dishes in 10% FCS-DMEM. When cells were 80% to 90% confluent, they were rinsed twice in PBS and then cultured in serum-free DCC for 24 hours. The confluent monolayer of cells was then wounded by scraping a narrow 200-μL pipetman tip across the plate in six parallel lines. Cells were rinsed twice in PBS and then grown in 5% DCC or in medium supplemented with E2 (10 nmol/L) or diarylpropionitrile (10 nmol/L). When indicated, cells were pretreated with PD98059 for 1 hour. After an additional 24 hours, each plate was examined by phase-contrast microscopy for the amount of wound closure by using Zeiss Axiovision 3.1 software to measure the physical separation remaining between the original wound widths. We made 18 separate measurements per plate and did each experiment in triplicate. Data represent the mean ± SE of three experiments.

Results

Protein expression profile of ERβ in HSG and HSY cells. To validate the previous findings that normal salivary gland and salivary duct adenocarcinoma specimens express only ERβ but not ERα (8), we analyzed the expression patterns of ERβ and ERα in salivary gland adenocarcinoma cell lines HSG and HSY. Western blot analysis revealed a single 57-kDa band corresponding to ERβ in both HSG and HSY cells. Neither of the two cell lines expressed ERα (Fig. 1A). Furthermore, immunofluorescent localization of ERβ in these cells revealed that ERβ was localized in both cytoplasmic and nuclear compartments (Fig. 1B).

Activation of MAPK and its upstream components by nongenomic signaling of ERβ. The exclusive expression of ERβ, and not ERα, in the HSG and HSY cells and the fact that ERβ was localized in both the nuclear and cytoplasmic compartments indicated that ERβ is the functional isoform in these cells, and that ligand stimulation may activate rapid nongenomic signaling pathways in these cell lines. To test this hypothesis, ligand-induced activation of the ERK/MAPK pathway was examined in HSG and HSY cell lines. As shown in Fig. 2A, both diarylpropionitrile and estrogen rapidly induced

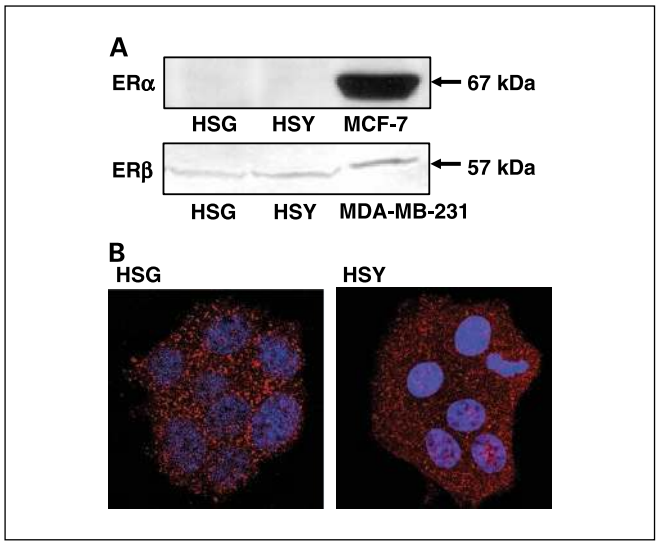

Fig. 1. ERα and ERβ expression in HSG and HSY cells. *A*, Western blot analysis of ERα and ERβ in HSG, HSY, MCF-7, and MDA-MB-231 cells. MCF-7 and MDA-MB-231 are positive controls for ERα and ERβ, respectively. *B*, immunofluorescent localization of ERβ (*red*) and DNA (*blue*) in HSG and HSY cells.

ERK phosphorylation in both HSG and HSY cells. The ligand-induced ERK/MAPK activation was not due to a direct effect of ligand on total ERK content because no changes in the total ERK expression level were detected after reprobing the membranes with a total ERK1/2 antibody. These results indicate that both diarylpropionitrile and estrogen activates nongenomic signaling in HSG and HSY cells.

To validate the above results and to further rule out the possibility that the activation of MAPK is not due to ERβ-mediated genomic signaling, we stimulated the cells with ER ligand estren, a nongenotropic signaling activator (4-estren-3α, 17β-diol; ref. 13). Results showed that upon stimulation with estren, there is a significant increase in MAPK activation at 30 minutes in both HSG and HSY cells (Fig. 2B).

We then examined the role of ERβ as a mediator of E2-triggered rapid ERK/MAPK activation by using the pure antiestrogen ICI 182780 as a competitive inhibitor of ERβ-mediated signaling. We treated exponentially growing HSG and HSY cells with ICI (100 nmol/L) for 1 hour before treatment, then the phosphorylation status of ERK1/2 was assessed by Western blotting. Treatment with ICI reduced E2-induced ERK/MAPK phosphorylation in both HSG and HSY cells (Fig. 3A). Of particular, the observed stimulation of MAPK by ligand was effectively blocked by PD98059, a widely used inhibitor of MAP/ERK kinase, which is an upstream activator of MAPK (Fig. 3A). These results suggest a role for the direct upstream components of MAPK in its stimulation by E2, further implicating the classic pathway of nongenomic signaling mediated by ERβ in both HSG and HSY cells.

Because the classic pathway of nongenomic signaling of ER involves the activation of upstream components of the MAPK pathway leading to Src homology and collagen/Src/Ras/ERK activation (2), we hypothesized that ligand treatment stimulates the upstream components of this pathway, such as Src, to activate MAPK. To test this hypothesis, we analyzed the activation status of Src in HSG and HSY cells stimulated by diarylpropionitrile and E2. Results showed that both diarylpropionitrile and E2 induced Src phosphorylation in both cell lines (Fig. 3B). Because Src has been shown to interact with and phosphorylate FAK on tyrosine residues acting as a docking site for growth factor receptor binding protein 2, which permits signaling to the RAS-MAPK cascade (14), we next analyzed the activation of FAK in these cell lines, and as expected, both diarylpropionitrile and E2 triggered FAK phosphorylation (Fig. 3B).

Because the FAK-Src signaling complex recruits and/or phosphorylates a number of signaling proteins and is involved with adhesion regulation and the motile and invasive phenotype, as well as with growth and survival signaling (15), we next analyzed whether the ligand-induced changes in Src and FAK activation were translated into dynamic cell morphologic alterations indicative of a more motile phenotype. Confocal microscopic analysis revealed that 24 hours of E2 treatment induced long cytoplasmic extensions, filopodia formation, and abnormal outgrowths in both HSG and HSY cells compared with the control cells (Fig. 4).

Involvement of E2-induced nongenomic signaling of ERβ with cytoskeletal changes and cell migration. To provide

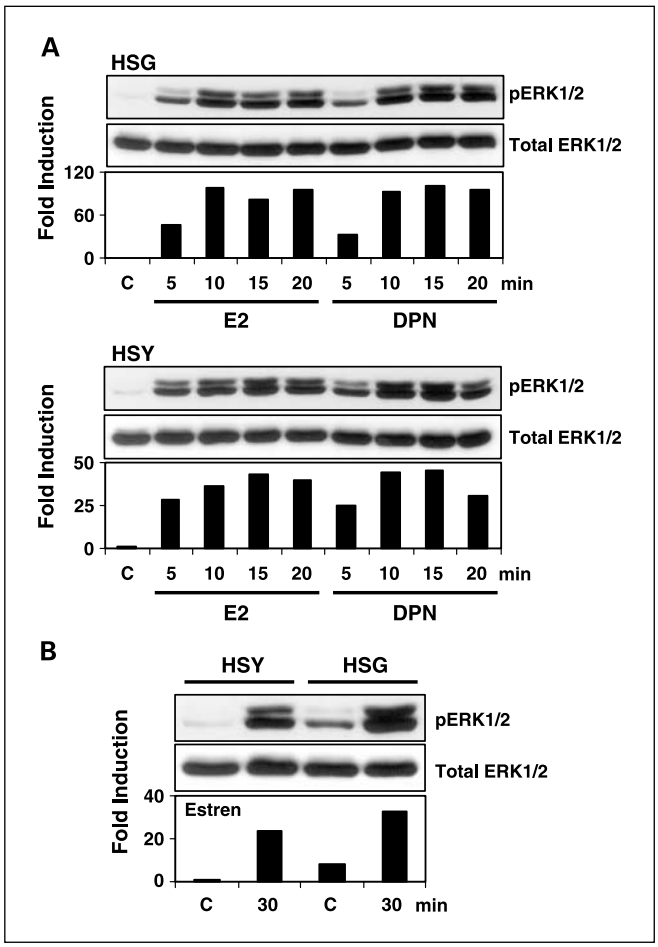

Fig. 2. ER ligands induced ERK1/2 phosphorylation in HSG and HSY cells. HSG and HSY cells were maintained in 1% DCC for 48 hours and then treated with (*A*) E2 (10 nmol/L) or diarylpropionitrile (*DPN*; 10 nmol/L) and (*B*) estren (10 nmol/L) for the indicated times. ERK1/2 phosphorylation was analyzed by Western blotting.

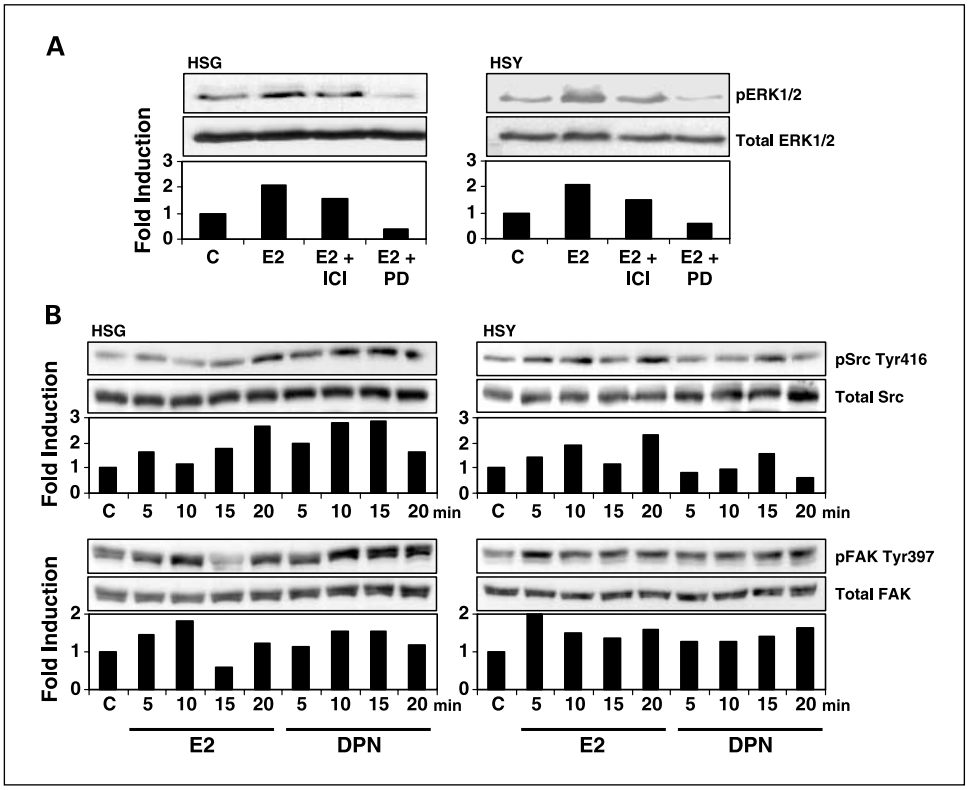

Fig. 3. Effect of ICI 182780 and PD98059 on E2-induced ERK1/2 phosphorylation (*A*) and E2 on Src and FAK phosphorylation (*B*) in HSG and HSY cells. *A,* HSG and HSY cells were maintained in 1% DCC for 48 hours and then pretreated with or without ICI 182780 (100 nmol/L) or PD98059 (10 μmol/L) for 1 hour before treatment with or without E2 (10 nmol/L). ERK1/2 phosphorylation was analyzed by Western blotting. *B,* HSG and HSY cells were maintained in 1% DCC for 48 hours and then treated with E2 (10 nmol/L) or diarylpropionitrile (10 nmol/L) for the indicated times. Src and FAK phosphorylation was analyzed by Western blotting.

proof-of-principle evidence that the observed phenotypic changes due to E2 induction on the actin cytoskeleton correlated with a functional physiologic process, we used a Boyden chamber and an established wound-healing assay to assess the migration of E2-stimulated HSG and HSY cells. Serum-starved cells showed a basal migration in the Boyden chamber, whereas overnight treatment with E2 resulted in a substantial increase in the migration of both cell lines (Fig. 5A). Similar results were obtained when ligand-dependent migration was assessed by a wound-healing assay (Fig. 5B). Furthermore, the observed increase in ligand-induced cell migration was effectively blocked by pure antiestrogen ICI 182780 (data not shown) and by MAP/ERK kinase inhibitor PD98059, clearly indicating that the E2-stimulated migration of HSG and HSY cells was mediated by the activation of ERK1/2, further confirming the involvement of the classic pathway of ER nongenomic signaling (Fig. 5C).

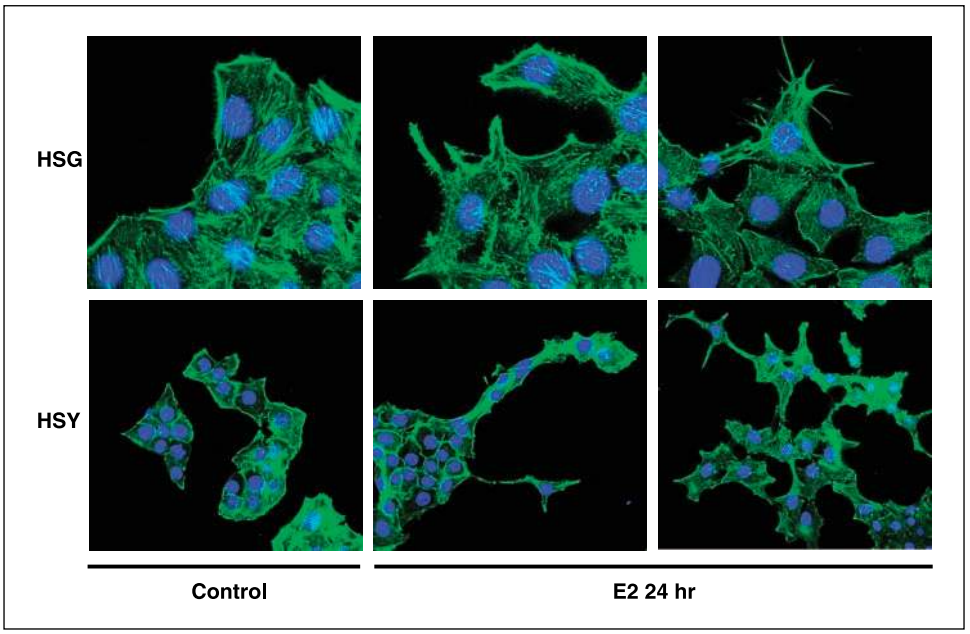

Fig. 4. E2 induces morphological changes in HSG and HSY cells. HSG and HSY cells were maintained for 48 hours in 1% DCC and then treated with E2 (10 nmol/L) for 24 hours. Cells were fixed and immunofluorescent labeled with fluorescently conjugated phalloidin (for filamentous actin) and ToPro3 (for DNA).

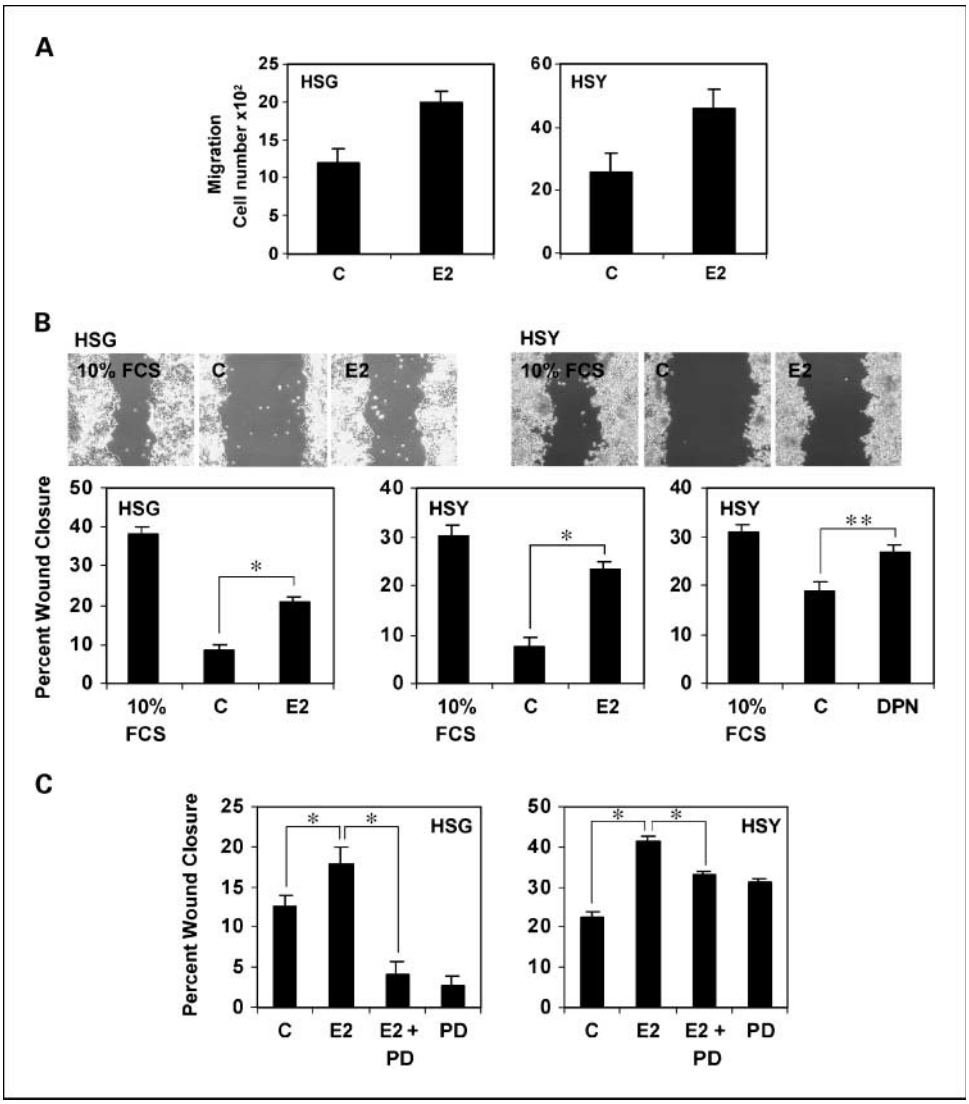

Fig. 5. Effect of E2 on cell migration in HSG and HSY cells. *A,* Boyden chamber cell migration assay was done as detailed in Materials and Methods. HSG and HSY cells were treated overnight with E2 (10 nmol/L). Experiments were done in triplicate. Columns, mean of the migrated cells; bars, SE. *B,* confluent 60-mm dishes of HSG and HSY cells were cultured in serum-free DCC medium for 24 hours, then the confluent monolayer of cells was wounded by scraping. Cells were then incubated with or without E2 (10 nmol/L) or diarylpropionitrile (10 nmol/L) in 5% DCC for 24 hours, and each dish was examined by phase-contrast microscopy for wound closure. *C,* HSG and HSY cells were cultured, and wound was created as above. Cells were then incubated with or without PD98059 (10 μmol/L) for 1 hour before treatment with or without E2 (10 nmol/L) in 5% DCC for 24 hours. Eighteen measurements were separately taken per dish, and each experiment was done in triplicate. Columns, mean of these experiments; bars, SE. *, $P < 0.001$; **, $P < 0.01$.

Discussion

Our findings showed that HSG and HSY cell migration resulted from ligand-induced ERβ nongenomic signaling through ERK1/2 activation, thus establishing the critical role of ligand-induced ERβ nongenomic signaling in the pathobiology of salivary gland carcinogenesis.

In recent times, a number of nonclassic estrogen-regulated tissues have been shown to express ERβ, suggesting that the function of these tissues is controlled by estrogen binding specifically to this ER subtype. ERβ is the predominant ER subtype in certain salivary gland carcinomas, and we now provide a further credence to these findings in both salivary gland adenocarcinoma cell lines HSG and HSY. It was previously established that the cytoplasmic localization of PELP1 promotes the nongenomic signaling of ER, which has been linked with the rapid responses to estrogen and generally involves stimulation of Src, MAPK, phosphatidylinositol 3-kinase, and protein kinase C pathways in cytosol (16). The selective expression of ERβ and not ERα, combined with the fact that PELP1 was predominantly cytoplasmic and coexpressed with ERβ in most salivary duct carcinomas, prompted us to examine the nongenomic signaling mechanism triggered by the ligand-ERβ complex and to explore the role played by these rapid signals.

A number of studies have shown that presence of ERβ in the plasma membrane, cytoplasm and mitochondria, in addition to its nuclear localization, supporting its possible involvement in nongenomic signaling (5, 7). We showed that nongenomic signaling, including ERK1/2, Src, and FAK, is activated by diarylpropionitrile and E2 in both HSG and HSY cells, which could be attributed to the membrane or cytoplasmic localization of ERβ. Of particular interest, preferential expression of the ERβ isoform has also been reported in platelets and has been shown to activate Src kinase via nongenomic actions (7). These ligand-induced dynamic changes in cytoplasmic signaling cascades were translated into filamentous-actin cytoskeletal rearrangements, adoption of motile cell phenotypes, and increased ability of stimulated cells to migrate. The classic pathway of nongenomic signaling of ER involves both upstream and downstream molecules of Src that have been previously linked to cell motility and cell migration. Our findings provide evidence of the formation of long cytoplasmic extensions,

filopodia formation, and abnormal outgrowths in both HSG and HSY cells upon E2 stimulation that seem to result from enhanced cell motility. Our findings were supported by our previous study that showed that E2 induced cytoskeletal remodeling and the migration of endometrial cancer cells by the activation of Src, FAK, and ERK1/2 (12).

To our understanding, this is the first report of the ability of the ERβ isoform in salivary gland adenocarcinoma cells to activate specific signal transduction pathways starting from the cytoplasm or plasma membrane, which may explain the effect of E2 in the modulation of cytoskeletal remodeling and the migration of salivary gland carcinoma cells.

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