Research Article

Estrogens and Human Papilloma Virus Oncogenes Regulate Human Ether-à-go-go-1 Potassium Channel Expression

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Abstract

Ether-à-go-go-1 (Eag1) potassium channels are potential tools for detection and therapy of numerous cancers. Here, we show human Eag1 (hEag1) regulation by cancer-associated factors. We studied hEag1 gene expression and its regulation by estradiol, antiestrogens, and human papillomavirus (HPV) oncogenes (E6/E7). Primary cultures from normal placentas and cervical cancer tissues; tumor cell lines from cervix, choriocarcinoma, keratinocytes, and lung; and normal cell lines from vascular endothelium, keratinocytes, and lung were used. Reverse transcription-PCR (RT-PCR) experiments and Southern blot analysis showed Eag1 expression in all of the cancer cell types, normal trophoblasts, and vascular endothelium, in contrast to normal keratinocytes and lung cells. Estradiol and antiestrogens regulated Eag1 in a cell type–dependent manner. Real-time RT-PCR experiments in HeLa cells showed that Eag1 estrogenic regulation was strongly associated with the expression of estrogen receptor-α. Eag1 protein was detected by monoclonal antibodies in normal placenta and placental blood vessels. Patch-clamp recordings in normal trophoblasts treated with estradiol exhibited potassium currents resembling Eag1 channel activity. Eag1 gene expression in keratinocytes depended either on cellular immortalization or the presence of HPV oncogenes. Eag1 protein was found in keratinocytes transfected with E6/E7 HPV oncogenes. Cell proliferation of E6/E7 keratinocytes was decreased by Eag1 antibodies inhibiting channel activity and by the nonspecific Eag1 inhibitors imipramine and astemizole; the latter also increased apoptosis. Our results propose novel oncogenic mechanisms of estrogen/antiestrogen use and HPV infection. We also suggest Eag1 as an early indicator of cell proliferation leading to malignancies and a therapeutic target at early stages of cellular hyperproliferation.[Cancer Res 2009;69(8):3300–7]

Introduction

Ether à-go-go (EAG) potassium channels possess oncogenic properties (1): human Eag1 (hEag1) mRNA shows restricted distribution in healthy tissues expressed mainly in brain; slightly in placenta, testis, and adrenal gland; and transiently in myoblasts (1–3). Interestingly, Eag1 is abundantly expressed in tumor cells, including cervical, lung, breast, colon, and prostate cancer (2, 4); therefore, Eag1 is suggested as a cancer marker. In addition, an association of Eag1 amplification with reduced overall survival has been observed in patients with colon carcinoma (5). Inhibition of Eag1 gene expression decreases tumor cell proliferation (1, 6, 7), and inhibition of Eag1 channel activity by imipramine or astemizole has been suggested to decrease cancer cell proliferation (6–8). Specific block of Eag1 with monoclonal antibodies inhibits tumor cell growth both in vitro and in vivo (9). Thus, Eag1 is also suggested as a cancer therapeutic target. Because no obvious side effects were observed in the animals treated with monoclonal antibodies (9), the potential therapeutic value of Eag1 channels seems to be very high.

High-risk human papillomavirus (HR-HPV) is the major risk factor for cervical cancer (10), and estrogen use is suggested as a likely factor contributing to this cancer (11). Actually, estrogen receptors (ER) or aromatase enzymes provide advantages for proliferation of tumor cells derived from mammary gland, lung, and cervix (12, 13). Nevertheless, tamoxifen stimulates proliferation of some cancer cells (14–16).

We reported Eag1 expression in normal cervical samples from women with negative pap smears, including a patient with HPV infection (4). In addition, Eag1 mRNA was detected in the tumor-free mammary gland surrounding the breast carcinoma tissue (2), in human diverticulitis (which has the potential to change into colonic cancer), and in crypt cells of a colon cancer mouse model (5). These observations suggest Eag1 expression as an early sign of cellular hyperproliferation.

Despite the potential clinical relevance of Eag1, its regulation by cancer etiologic factors is poorly understood. Here, we studied hEag1 expression and regulation by some cancer-associated factors, namely, estrogens, antiestrogens, and HPV oncogenes in normal and tumor cells. We studied cervical cancer cells obtained from primary cultures and cell lines from different histogenesis.
Materials and Methods

Biological samples. Biopsies from normal human placentas (38–42 wk of gestation, n = 7) were obtained from patients registered at Hospital "Gabriel Mancera" following the local ethical considerations and used to obtain normal keratinocytes primary cultures.

Cell culture and reagents. Cytotrophoblasts were isolated and cultured as described (18, 19). Villous tissue was enzymatically dispersed; cytrophoblasts were separated on a Percoll gradient, plated in DMEM-HG with 20% fetal bovine serum (FBS) and incubated at 37°C (95% humidity and 5% CO2 atmosphere). Human chorionic gonadotrophin hormone (hCG) concentration was measured with an immunoassay kit (Immunometrics Ltd.). Human cervical cancer cells were obtained from previously established primary cultures (4). Tumor cell lines (HeLa, SiHa, CaSki, INBL, and C33A from cervix; JEG-3 and A-549 from choriocarcinoma and lung, respectively), as well as bronchial epithelial cells (BEAS) and human umbilical vessel endothelial cells (HUVEC) from normal lung and umbilical cord, respectively, were obtained from American Type Culture Collection and cultured according to manufacturer's instructions. Cell treatments were performed, as follows, to avoid participation of exogenous estrogens present in serum or medium: cells were washed with HBSS 24 h after plating and incubated in supplemented medium (DMEM-HG without phenol red plus 5% charcoal-stripped FBS and antibiotics). Cells were incubated with 17β-estradiol (Sigma Chemical Co.) and/or the antiestrogens ICI 182,780 (Geneca Pharmaceuticals) or 4-hydroxytamoxifen (Sigma Chemical Co.) during 24 h; ethanol was used as vehicle, and incubations were stopped by aspirating the culture media and adding Trizol reagent (Invitrogen) for RNA extraction. Normal keratinocytes were prepared from neonatal foreskins and grown in the keratinocyte serum-free medium (Invitrogen) supplemented with 20 mg/mL bovine pituitary extract and 0.1 ng/mL of epidermal growth factor (Invitrogen); transformed human keratinocytes (HaCat cells) provided by Dr. Lutz Gissmann (German Cancer Research Center (DKFZ)) were cultured in the same medium. Primary human keratinocytes immortalized by amphotrophic retroviruses encoding E6, E7, or E6/E7 oncoproteins of HPV-16 (provided by Dr. Frank Rösl, DKFZ) were grown in a keratinocyte growth medium (Invitrogen) and cultured as described (20).

Transient human ER-α transfection and ER Western blot. Expression vector for human ER-α (pCMVα-hERα) to transfected HeLa cells was provided by Dr. Aj Cooney (Baylor College of Medicine). Cells were transfected using PolyFect (Qiagen, Inc.) as described (21). Transcriptional activity of transfected ERα was confirmed by a chloramphenicol acetyltransferase (CAT) reporter assay (21). Western blots were performed to detect ERα in HeLa, A-549, BEAS, and cervical cancer cells with the primary antibody anti-ERα (Santa Cruz). HeLa cells expressing ERα (HeLa-ERα) were subjected to the same treatments as described above.

PCR amplifications and Southern blot analysis. Total RNA was isolated from tissues and cell cultures with Trizol reagent. RNA was reversely transcribed, and PCR amplifications were performed with the following primers: 5′-GCTTTTGGAGGTGGATGAG-3′ and 5′-CGAGGAGGATGAGATGAGTG-3′, which yielded 475-bp hEag1 reverse transcription-PCR (RT-PCR) products. In some cases, Eag amplification was performed using the following sense and antisense primers: 5′-TGGCTCTGCTGTTGTTGGTG-3′ and 5′-ACACAGGGAGAGATGAGACG-3′. These amplifications yielded a 187-bp hEag1 product. Human Eag1-transfected Chinese hamster ovary (CHO) cells (provided by Walter Stühmer, Max-Planck-Institut für Experimentelle Medizin) were used as positive control. Cyclophilin and β2-microglobulin genes were used as constitutive genes and amplified with the following primers: 5′-CCC CAC CCA GGT GCT ACC CAT-3′ and 5′-AGG TCC TTA CCG TTC TGG TCG-3′ for cyclophilin yielding a 453-bp product and 5′-ACCC CACTGAAA-3′ and 5′-ATGATGCTGCTACTCATGCTGCTGAT-3′ for β2-microglobulin yielding a 100-bp product. Identity of the 475-bp Eag RT-PCR product has been already determined by nucleotide sequence (4). For Southern blot analysis, PCR products were separated in agarose gels, blotted onto nylon membranes, and hybridized with [32P]dCTP-labeled nested probes. Probes were obtained

In the case of normal cells/tissues, we selected some normal cell lines and normal human placenta as a very interesting tissue to study, because placental development resembles tumor growth (17) and estrogens play a pivotal role in placental physiology, and noteworthy, because it expresses Eag1 mRNA and the potential role of Eag1 in healthy tissues is almost unknown. Our regulation studies unravel potential oncogenic mechanisms of HPV infection and estrogen/antiestrogen use, emphasizing the potential use of Eag1 as an early indicator of cell proliferation. On the other hand, our pharmacologic experiments suggest Eag1 as a therapeutic target at the early stages of cell hyperproliferation.

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"La Raza," following the local ethical considerations. Tissue was placed in liquid nitrogen to obtain mRNA or fixed in paraformaldehyde for immunohistochemistry. For cytrophoblast culture, tissue was washed with saline solution and immediately processed. Neonatal foreskins from patients registered at Hospital "Gabriel Mancera" were collected following the local ethical considerations and used to obtain normal keratinocytes primary cultures.

Materials and Methods

In the case of normal cells/tissues, we selected some normal cell lines and normal human placenta as a very interesting tissue to study, because placental development resembles tumor growth (17) and estrogens play a pivotal role in placental physiology, and noteworthy, because it expresses Eag1 mRNA and the potential role of Eag1 in healthy tissues is almost unknown. Our regulation studies unravel potential oncogenic mechanisms of HPV infection and estrogen/antiestrogen use, emphasizing the potential use of Eag1 as an early indicator of cell proliferation. On the other hand, our pharmacologic experiments suggest Eag1 as a therapeutic target at the early stages of cell hyperproliferation.

Materials and Methods

Biological samples. Biopsies from normal human placentas (38–42 wk of gestation, n = 7) were obtained from patients registered at Centro Médico

Figure 1. Eag1 channels in human placenta. A. Eag1 protein was immunolocalized (dark blue-violet staining) in the cytoplasm, nucleus of the syncytiotrophoblast layer (SC), and nucleus of vascular endothelial cells (VEC). Magnification, 200×. Insert, negative control in the absence of Eag1 antibody. Image is representative of seven placentas. In some other sections, Eag1 was also expressed in the plasma membrane (data not shown). B. Southern blot analysis (top) from RT-PCR experiments of seven different normal-term placentas. All of them displayed Eag1 expression. Columns, Eag1 mRNA relative expression after normalization with the constitutive gene cyclophilin (cyc).
as previously described and also confirmed by sequence (4). In all cases, filters were washed after 18 h of hybridization and exposed to X-ray films.

**Real-time PCR amplifications.** SYBR Green real-time PCR was performed with 1 μL of cDNA obtained as described above. SYBR Green reaction was conducted using a QuantiTect SYBR Green PCR reagents kit (Qiagen) following the manufacturer's instructions. The following primers were used: 5′-TGG TCC TGC TGG TGT GTG-3′ (forward), 5′-ACA AGG AGA TGT AGA CAG-5′ (reverse) for Eag1 (4) and 5′-ACC CCC ACT GAA AAA GAT GAG TAT-3′ (forward), 5′-ATG ATG CGT TTG ACA TGG CTC GAT-3′ (reverse) for β2-microglobulin (used as constitutive gene and to analyze relative Eag1 expression). Data analysis was performed by the 2^{-ΔΔCt} method.

**Eag1 immunochemistry.** Specific anti-Eag1 antibodies were kindly provided by Walter Stühmer and Luis Pardo (Max Planck Institute). This antibody has been validated and shown to be very specific; it does not bind to hErg channels and discriminates between Eag1 and Eag2 (9). With this antibody, we already reported Eag1 channel expression in human cervical biopsies, cervical cancer cells from primary cultures, and CHO cells transfected with Eag1 in opposite to the absence of Eag1 in nontransfected CHO cells (4). Placental biopsies were fixed in a buffered ethanol. After washing with TBS/Tween (pH 7.4), slides were immersed in EDTA buffer [0.01 mol/L (pH 8.0)] and boiled for antigen retrieval. Slides were blocked for 2 h with levamisole and incubated in the presence of 1:50 anti-Eag1 antibody (I0001 single-chain antibody coupled with alkaline phosphatase) for 2 h at room temperature. The slides were washed with TBS/Tween, and the specific staining reaction was completed by incubating the slides in the presence of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Roche) in a buffer solution for 1 h at room temperature, protected from light, and observed as a dark blue-violet staining. Sections were counterstained with eosin. Slides were observed with a Nikon Eclipse E600 microscope using a Nikon Plan Apo 10×/0.45 objective lens. Images were acquired with a DMX1200 camera and the Nikon ACT-1 acquisition software at room temperature. Keratinocytes expressing HPV oncoproteins (K-E6, K-E6/E7) were processed in a very similar manner. After antigen retrieval with EDTA, slides were blocked with endogenous peroxidase and phosphatase blocker (Dako) for 10 min and then incubated in the presence of 1:100 anti-Eag1 antibody for 1 h at room temperature. The slides were then incubated with MACH 4 mouse probe (Biocare) in TBS/Tween for 10 min and then incubated with MACH 4 horseradish peroxidase polymer (Biocare) in TBS/Tween for 10 min. The specific staining reaction was completed by incubating the slides in the presence of diaminobencidine in buffer reaction solution (Dako) and observed as a brown staining. Sections were counterstained with hematoxylin (Dako), observed with a Nikon Eclipse E600 microscope using a Nikon Plan Apo 40× objective lens, and analyzed as above described for the placental sections.

Figure 2. Eag1 regulation by E2 in cultured human syncytiotrophoblast and vascular endothelial cells. Southern blot analysis from RT-PCR experiments shows that E2 induced Eag1 mRNA expression in syncytiotrophoblasts with no detectable basal Eag1 expression (A) and antiestrogenic treatment with ICI 182,780 (ICI) prevented such up-regulation and decreased Eag1 levels in a syncytiotrophoblast primary culture with basal Eag1 expression (B). Eag1 is endogenously expressed in HUVEC and up-regulated by E2, and antiestrogenic treatment prevented such up-regulation (C) as in trophoblasts. Columns, Eag1 mRNA relative expression after normalization with cyclophilin (A–C); Eag1 expression in vehicle was given the value of 1 in B and C. D, whole-cell patch-clamp experiments showed outward potassium currents in syncytiotrophoblasts treated with E2 (top) and untreated choriocarcinoma JEG-3 cells (bottom). Currents elicited at +60 mV were preceded by negative prepulses (−140 or −60 mV) and displayed different activation kinetics, suggesting Eag1 channel activity. Eh = −80 mV, unsubtracted traces. Figures are representative of at least three separate experiments.
alone or in the presence of a stemizole (10 μM). Mannheim GmbH. Cells were incubated during 96 h in culture medium 2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit I, Boehringer dehydrogenase activity inactivates mitochondria [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by formazan crystals by colorimetric method based on the conversion of the tetrazolium salts to formazan crystals by mitochondrial dehydrogenase activity.]

Eag1 up-regulates experimentally performed at room temperature (20–22°C). Holding potential was −80 mV, unless indicated. Experiments were performed at room temperature (20–22°C).

Electrophysiology. Whole-cell recordings were acquired from isolated cells with the patch-clamp technique (22) using an EPC-9 amplifier (HEKA Electronics) and analyzed with Igor Pro (WaveMetrics). Patch pipettes (2–3 MD) were obtained by double-pulling Kimax capillaries. Internal solution contained (in mmol/L) 140 KCl, 10 EGTA, and 10 HEPES/KOH (pH 7.2). External solution contained (in mmol/L) 2.8 KCl, 115 NaCl, 2 CaCl2, 2 MgCl2, and 10 HEPES/NaOH (pH 7.2); in some experiments, we used external solutions containing 10 mmol/L MgCl2. Holding potential was −80 mV, unless indicated. Experiments were performed at room temperature (20–22°C).

Metabolic activity and apoptosis. K-E6/E7 cells were seeded in 96-well plates, and cell proliferation was assayed by the colorimetric method based on the conversion of the tetrazolium salts to formazan crystals by dehydrogenase activity in active mitochondria [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit I, Boehringer Mannheim GmbH]. Cells were incubated during 96 h in culture medium alone or in the presence of astemizole (10 μmol/L), imipramine (10 μmol/L), DMSO as vehicle (all of these reagents were purchased from Sigma Chemical Co.), or the anti-Eag1 monoclonal antibody 56 (133 nmol/L, also provided by Walter Stühmer and Luis Pardo) known to inhibit Eag1 channel activity (9). MTT (0.5 mg/mL) was added 4 h before completing the whole incubation time. Absorbance data were obtained from the resulting colored solution with a microplate photometer (Sunrise Touchscreen). Apoptosis was determined with the Annexin V-FITC kit (Invitrogen Co.) binding to phosphatidylserine and DNA staining by propidium iodide (PI). Camptothecin (apoptosis inducer) and methanol (necrosis inducer) were used as controls. Experiments were carried out with the flow cytometer FACS-SYAN ADP with nine colors (DAKO). Percentages of viable (FITC and PI negative), apoptotic (FITC positive and PI negative), and late apoptotic (FITC and PI positive) cells were obtained by quadrant analysis using WinMDI software version 2.8.

Statistical analysis. Student's t test or ANOVA, followed by Dunnett's or Tukey-Kramer test, was used to compare data between different groups; P values of <0.05 or <0.01 for the respective tests were considered to be statistically significant. Analysis was made using GraphPad Prism software version 3.0.

Results

Regulation of Eag1 by Estradiol in Normal and Tumor Cells

Eag1 channels in normal human placenta and vascular endothelium and their regulation by estradiol. We investigated Eag1 protein expression in normal human placenta and studied the likely regulation of Eag1 gene expression by 17β-estradiol (E2) in primary cultures from normal human trophoblasts. Eag1 channels were strongly expressed in the syncytiotrophoblast layer in both the cytoplasm and the nucleus in normal placenta (Fig. 1A, dark blue-violet staining). Interestingly, we also observed Eag1 channels in the vascular endothelium, especially in the nucleus (Fig. 1A). Whereas Eag1 protein expression pattern was very similar among placentas, relative Eag1 mRNA levels were very different. Figure 1B shows some placentas with low Eag1 mRNA expression and some with higher levels. Despite such differences in mRNA levels, we observed both Eag1 protein and mRNA expression in all of the placentas studied.

Unexpectedly, Eag1 mRNA was not detected in most of the trophoblasts primary cultures but was induced by estradiol. Figure 2A shows Eag1 mRNA up-regulation by E2 at picomolar concentrations whereas a higher E2 concentration originated only a faint Eag1 signal. The antiestrogen ICI 182,780 prevented such up-regulation. In cultures displaying endogenous Eag1 mRNA (Fig. 2B), E2 induced a slight increase in Eag1 expression and antiestrogen treatment down-regulated endogenous Eag1 expression both in the presence and absence of E2 (Fig. 2B). As expected, we also found endogenous Eag1 expression in the placental choriocarcinoma cell line JEG-3; however, in this case, we did not find a significant estrogenic regulation (data not shown).

Eag1 channel expression in vascular endothelium prompted us to study Eag1 mRNA expression and regulation by estradiol in endothelial cells. Endogenous Eag1 mRNA was found in normal HUVECs supplemented with 10% FBS (Fig. 2C). Eag1 was up-regulated by E2; this effect was inhibited by ICI 182,780, which also down-regulated endogenous Eag1 levels (Fig. 2C). The effect of estrogens and antiestrogens on Eag1 expression was very similar in both normal cell types (trophoblasts and vascular endothelium).

Endogenous Eag1 channel activity has not been reported for most of the normal tissues where it is expressed, including the brain. Therefore, we investigated if Eag1 channel activity could be recorded in placental-derived cells. Whole-cell patch-clamp recordings showed no outward voltage-activated potassium currents in trophoblast primary cultures in the absence of E2 (data not shown). However, when incubated with E2 (1 pmol/L), normal human trophoblasts displayed noninactivating outward currents resembling Eag1 channel activity (Fig. 2D, top). Eag1 channels display a strong Cole-Moore shift (the current elicited with a depolarizing pulse is slower at a more negative prepulse voltage) and a very slow
activation in the presence of extracellular magnesium (23). Figure 2D shows that outward currents elicited at +60 mV are slower when preceded by a very negative prepulse (−140 mV) compared with a more positive prepulse (−60 mV), recordings were obtained in the presence of 10 mmol/L MgCl₂. Because no other potassium channels have been reported to display such a strong Cole-Moore shift, our data suggest Eag1 channel activity in normal human trophoblasts incubated with E₂. Similar currents were elicited in the choriocarcinoma Jeg-3 cells in the absence of estradiol (Fig. 2D, bottom), although, in this case, the current was not that accelerated with a more positive prepulse, as in normal human trophoblasts, despite of a lower MgCl₂ concentration (2 mmol/L) used.

### Expression and Potential Role of Eag1 in Cells Expressing HPV Oncogenes

Eag1 in cervical cancer cell lines and keratinocytes expressing HPV oncoproteins. We studied the expression of Eag1 in normal keratinocytes and immortalized keratinocytes (data not shown). Therefore, we transfected these cells with the Eag1 cDNA, and studied again Eag1 regulation by E₂. ER-α-mediated transcription was confirmed by CAT assays (21) in HeLa-ERα cells cotransfected with a CAT reporter plasmid (data not shown). Figure 3B shows a remarkable Eag1 up-regulation by E₂ in HeLa-ERα cells. ICI 182,780 and tamoxifen, alone or in combination with E₂, produced a very high Eag1 up-regulation (Fig. 3B). These results suggest ERα activation as one of the mechanisms of the estrogenic regulation of Eag1.

Eag1 regulation was also studied in cervical cancer cells from three human cervical biopsies. Lung cell lines A549 and BEAS-2B from cancer and normal tissue, respectively, were used to study both Eag1 expression and estrogenic regulation in cells from a very common cancer influenced by estrogenic signaling (12) but histogenically different from placental or cervical tissue. Western blot analysis showed that cervical and lung cells express ERs (data not shown). Cervical cancer cells from primary cultures were previously characterized to express Eag1, HPV, and cdk2 (4), supporting their cancer epithelial nature. Table 1 shows screening of Eag1 estrogenic regulation in these cells. Effect of E₂ and antiestrogens on Eag1 expression in primary cultures from cervical cancer was similar between two patients (cervical cancer 1 and 2); interestingly, in a sample from another patient (cervical cancer 3), such regulation occurred in the opposite direction in all of the treatments studied. As expected, Southern blot analysis showed the absence of Eag1 gene expression in the normal human lung cells (BEAS-2B). Eag1 was not induced by E₂ or antiestrogens in these cells (data not shown). In contrast, Eag1 was present in the lung cancer cell line A549 and up-regulated with most treatments, especially by antiestrogens (Table 1). Thus, E₂ also regulates Eag1 expression in cancer cells in a cell-type–dependent manner. Then, we wondered whether a well-identified etiologic factor for some cancers could also affect Eag1 expression.

### Discussion

Eag1 is a potential tool for cancer detection and therapy (1, 2, 4, 9), and several findings suggest Eag1 as an early marker for breast, cervical, and colon cancer (2, 4, 5).
Figure 4. Eag1 in cervical cancer cells and effect of Eag1 inhibitors in cell proliferation and apoptosis of keratinocytes expressing HPV oncogenes. A, duplex PCR experiments and densitometric analysis for Eag1 and β2-microglobulin showed no Eag1 expression in normal keratinocytes (NK); in contrast, keratinocytes expressing HPV oncogenes (K-E6, K-E7, or both, K-E6/E7) expressed Eag1. Eag1 was also found in transformed keratinocytes without HPV (HaCat and C33A) and in cervical cancer cell lines with either HPV-16 (CaSki and SiHa) or HPV-18 (HeLa and INBL). Columns, mean of Eag1 mRNA relative expression after normalization with β2-microglobulin; bars, SD. Eag1 expression in HeLa cells was given the value of 1. B, Eag1 protein (brown staining) in K-E6/E7 and K-E6 cells can be observed in the plasma membrane (CM), nuclear membrane (Nuc), cytoplasm (Cyt), and densitometric analysis for Eag1 protein (brown staining) in the insets. Magnification, 800×. C, nonspecific Eag1 inhibitors astemizole (Astem) and imipramine (Imp; 10 μmol/L, 96 h) decreased cell proliferation of keratinocytes expressing both HPV oncogenes (K-E6/E7), assayed by metabolic activity; the anti-Eag1 monoclonal antibody 56 (mAb56), a very specific inhibitor of Eag1, also decreased metabolic activity. D, astemizole increased apoptosis in these K-E6/E7 cells. Columns, mean; bars, SD. *, P < 0.05 compared with control (medium or vehicle).

Placental development resembles malignant tumor behavior (17). We found Eag1 protein in the syncytiotrophoblast layer and vascular endothelium of placental villi at the plasma membrane, in the cytoplasm, and in the nucleus. This distribution was expected because Eag1 has a bipartite nuclear targeting signal in its carboxy terminus (24) and perinuclear localization of Eag1 channels has been observed in tumors (2, 6). A COOH terminal fragment of a voltage-gated calcium channel translocates to the nucleus and regulates transcription (25), as a similar role for Eag1remains elusive. Nevertheless, Eag1 mRNA expression was barely detected in trophoblast primary cultures despite cell viability confirmation by basal and 8-bromo-cyclic AMP–stimulated placenta hCG secretion (data not shown). Conversely, Eag1 mRNA expression was induced by E2. Choriocarcinoma JEG-3 cells displayed endogenous Eag1 gene expression; however, Eag1 expression was only slightly regulated by estrogens and unaffected by ICI 182,780. Our results suggest that Eag1 might participate in the proliferation and/or fusion of trophoblast (as in myoblasts fusion; ref. 3), but this deserves further investigations. Eag1 protein was found in placental blood vessel endothelium, accordingly; endogenous Eag1 mRNA was found in normal HUVECs, up-regulated by E2 and down-regulated by ICI 182,780. This is important because estradiol is also considered as an angiogenic factor (26–28). Interestingly, it has been recently found that Eag1 promotes vascular endothelial growth factor secretion and angiogenesis in tumors (29). The effect of other angiogenic factors on Eag1 expression in both normal and cancer cells remains to be elucidated. We show the first recordings suggesting Eag1 channel activity in normal human trophoblasts, but more pharmacologic studies are needed to determine the potential contribution of other potassium channels to the outward currents recorded in placental cells.

Human ERα is necessary for a clear estrogenic regulation of Eag1 gene expression in HeLa cells. Only when forced to express ER-α did estradiol and antiestrogens clearly up-regulated Eag1. Actually, this is a potential explanation for the lack of estrogenic regulation of Eag1 in JEG-3 cells, because these cells express very low amounts of ER-α (30, 31). Thus, concerning ER-α expression, JEG-3 cells could be compared with HeLa wild-type cells not transfected with ER-α. When screening estrogenic regulation of Eag1 mRNA in cells from cervical cancer primary cultures derived from three patients, we found similar effects in two cases but opposite effects in another culture. Accordingly, tamoxifen opposite effects have been reported in cervical cancer. Tamoxifen treatment inhibited growth of cervical cancer cell lines, including HeLa cells (32); but in the cervical cancer cell line SFR lacking ERs, tamoxifen stimulated HPV-16 gene expression and cell proliferation (16). Cervical cancer biopsies
(16%) from patients treated with tamoxifen had a significant decrease in the number of mitotic figures (33). Thus, the variable estrogenic regulation of Eag1 in cervical cancer primary cultures might have several explanations, including treatment conditions, differences in HPV copy number, and different amounts of ERs or ER phosphorylation; actually, tamoxifen can switch from ERα antagonist to ERα agonist if ERα is phosphorylated (34).

Estrogens stimulate growth and progression of lung tumors (35), and aromatase inhibitors suppress tumor growth in mice with A-549 lung xenografts (12). Eag1 was not found in BEAS-2B cells in accordance with the absence of Eag1 in normal bronchial epithelium (2). We found Eag1 expression in A-549 cells in compliance with the findings in lung cancer biopsies (2); in these cells, almost all treatments up-regulated Eag1. A combined therapy of antiestrogens and epidermal growth factor receptor (EGFR) inhibitor has been suggested for lung cancer (36) because of the functional linkage between estrogens and EGFRs (37). Therefore, Eag1 up-regulation by antiestrogens might be related to the cross-talk between different signal transduction pathways.

Different cervical cancer cell types and keratinocytes expressing HPV oncogenes displayed Eag1 expression in contrast to normal keratinocytes. HPV oncoproteins E6 and E7 influence cell proliferation by targeting p53 and Rb proteins, respectively (38). The cell lines used present different molecular alterations. HeLa cells overexpress c-Myc and Bcl-2 proteins, SiHa cells have high levels of pRb and BclXL proteins (39), and keratinocytes expressing E7 display high c-Myc levels (20). Eag1 was also found in transformed keratinocytes lacking HPV (HaCat and C33A cells). Interestingly, p53 and Rb are mutated in C33A cells (40) and HaCat cells have two mutations in p53 (41). Probably Eag1 is down-regulated, at least, through p53 and Rb pathways. Because Eag1 was found in cells presenting or lacking HPV, these results suggest that Eag1 gene expression is regulated by different factors, which is in accordance with the Eag1 overexpression observed in very different types of tumors (2, 4–6); our results also offer possible alternative explanations for the oncogenic mechanisms of HPV infection. Eag1 protein was found in K-E6/E7 and K-E6 cells, and Eag1 channel inhibitors affected cell proliferation and apoptosis. Astemizole was more effective than imipramine in inhibiting cell proliferation and increasing apoptosis in keratinocytes expressing HPV oncogenes. Both drugs have other targets different from Eag1; thus, a plausible explanation is a higher affinity of other proteins participating in cell proliferation/apoptosis to astemizole. One of such proteins might be hERγ channels, which are strongly inhibited by astemizole and have also been suggested to play a role in tumorigenesis (42–43). Further mechanistic studies are required to understand the effect of astemizole on cell proliferation and apoptosis. Nevertheless, despite of some nonspecific effects of imipramine and astemizole that might be involved, the effect of these drugs and the monoclonal Eag1 antibody on proliferation and/or apoptosis suggests Eag1 as a target at early stages of cell hyperproliferation, for example, in cells initially transformed by HPV.

Eag1 channel activity is increased by arachidonic acid in IGR1 melanoma cells (46), and insulin-like growth factor-I increases both channel activity and gene expression of Eag1 in MCF-7 breast cancer cells (47). Here, we show for the first time Eag regulation by cancer risk factors in normal cells. A highlighted feature in this study was that Eag1 can be regulated by both estrogens and HPV oncoproteins. Eag1 up-regulation by E2 in HeLa cells and some cervical cancer primary cultures suggest that estrogens and HPV might have a synergistic effect on Eag1 expression. Actually, it has been shown that estrogens contribute to the onset, persistence, and malignant progression of cervical cancer in a HPV-transgenic mouse model (48). A very interesting issue to address is to know the potential participation of Eag1 channels in the mouse cervical cancer model mentioned. Another interesting issue is to investigate the differential concentration-dependent effect of estrogens. Concentration-dependent dual effects of estrogens have been already observed, for example, on growth hormone synthesis and interleukin-induced proteoglycan synthesis (49, 50).

Our experiments unravel novel potential oncogenic mechanisms of estrogen/antiestrogen use and HPV infection. This proposes that Eag1 might be expressed in early stages potentially leading to cervical transformation, for example, in patients infected with HPV or those undergoing estrogen treatment or subjects with low-grade intraepithelial lesions.

The data presented herein suggest Eag1 as an early marker of cellular proliferation leading to malignancies, enhancing potential benefits of using Eag1 in early detection programs. Because cancer is a multifactorial disease, detecting the expression of oncogenic genes or proteins regulated by several cancer risk factors, like Eag1, might lead to improved diagnosis methods and early detection of malignancies. Finally, our pharmacologic experiments propose Eag1 as a therapeutic target at early stages of cell hyperproliferation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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