Human EAG1 Potassium Channels in the Epithelial-to-Mesenchymal Transition in Lung Cancer Cells

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Abstract. *Background: Human ether à go-go-1 (EAG1) potassium channels are potential tools for cancer diagnosis, prognosis and therapy. Epithelial-to-mesenchymal transition (EMT) is a likely mechanism by which tumor cells become malignant. We wondered whether EAG1 is regulated in human lung tumor cells undergoing EMT. Materials and Methods: EMT was induced in A549 lung tumor cells with transforming growth factor beta (TGFβ1). EAG1 gene expression was assesed by real-time RT-PCR and protein expression by flow cytometry. Results: TGFβ1 produced the expected changes in morphology, migration and gene expression associated to EMT. EAG1 gene and protein expression were up-regulated during EMT. Astemizole did not prevent EMT. Conclusion: Our results suggest that EAG1 channels participate in the acquisition of a malignant phenotype in lung tumor cells. Their potential role in EMT might not be uniquely related to their conducting function, in accordance with the reported tumor growth supported by non-conducting EAG1 channels.*

Epithelial to mesenchymal transition (EMT) is a reversible cellular process characterized by the loss of cell polarity, morphological changes, down-regulation of adherent molecules, increased expression of mesenchymal markers and enhanced ability to migrate (1). EMT has been described during embryonic development, wound healing, tissue regeneration, fibrosis, and tumor progression (2). Accordingly, expression of mesenchymal markers (vimentin, fibroblast protein 1, SNAIL1 and SNAIL2, nuclear β catenin, stromelysin-3) and loss of epithelial markers correlate with poor prognosis (3). Moreover, invasion of adenocarcinoma is accompanied by the release of single cells through an EMT process (4). Thus, it has been proposed that cancer cells acquire a malignant phenotype through an EMT process (5).

The human voltage-gated potassium channel ether *à go-go* 1 (EAG1, Kv 10.1) is mainly expressed in normal brain, slightly in placenta, adrenal gland, testes, and transiently expressed in myoblasts at the onset of the fusion (6-8). In contrast, EAG1 is overexpressed in most types of tumors including leukemia (8-10). Inhibition of either activity or expression of EAG1 reduces tumor cell proliferation *in vitro* and *in vivo* (11). Interestingly, it has been shown that non-conducting EAG1 channels still support tumor growth of xenografted CHO cells transfected with *EAG1* (12).

There are only a few reports studying ion channels in EMT. It has been shown that the expression of bestrophin 1 (a calcium-regulated chloride channel found in tumor cells), is up-regulated during the EMT process of renal collecting duct cells (13). Some studies lead to the belief that EAG1 might be regulated during EMT. EAG1 expression is enhanced by the insulin-like growth factor 1 (IGF-1) in breast tumor cells (14). IGF-1 has been proposed as a promoter of EMT in human prostate cancer cells (15) and suggested to be involved in EMT of breast tumor cells (16). Moreover, transgenic mice that overexpress a constitutively activated type 1 insulin-like growth factor receptor in breast, developed several mammary tumors with apparent features of EMT (17). *De novo* expression of EAG1 has been observed in human keratinocytes transfected with the *E6/E7* oncogenes from human papillomavirus 16 (HPV-16) (18). Interestingly, E6 and E7 oncoproteins induce changes associated with EMT, such as low expression of E-cadherin and *de novo* expression of vimentin in human primary keratinocytes (19). Of note, *EAG1* gene amplification has been associated with poor prognosis in colon carcinoma (20) and a higher EAG1 expression has been found in fast growing T84 colon carcinoma cells in comparison to slowly growing carcinoma cells (21). In addition, it has been found that EAG1 expression is up-regulated in invasive breast carcinoma (22) and that inhibition of its activity decreases migration of leukemia cells (9), and impairs metastasis of lung tumor cells (10). In addition, when Chinese hamster ovary (CHO) cells transfected with *EAG1* are grown on different extracellular

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matrix components, an actin cytoskeleton rearrangement suggesting an active migration state is observed (23). EAG1 expression has been reported in lung carcinoma and A549 lung tumor cells (8, 18). EMT is induced by TGFβ1 in different cell types (24-26). EAG1 channels promote tumor cell proliferation and are regulated by factors associated to EMT. In addition, EAG1 has been suggested to participate in tumor malignancy. Therefore, here we studied EAG1 expression in lung cancer cells undergoing EMT induced by TGFβ1. This is the first report studying EAG1 expression in the acquisition of a malignant phenotype in tumor cells, emphasizing the potential clinical relevance of EAG1 channels.

Materials and Methods

Cell culture and reagents. A549 cell line (from human alveolar adenocarcinoma) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) plated in 75 cm^2 flasks with F12-K culture medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; PAA laboratories Inc., Dartmouth, MA, USA) and maintained following the manufacturer's instructions. In order to induce EMT, cells were plated in 60 cm^2 dishes in 0.5% FBS-F12 and treated with TGFβ1 (Sigma Chemical Co, St Louis, MO, USA) for 48 hours as described (24-26).

Semiquantitative and real-time RT-PCR. Total RNA was isolated with Trizol reagent. cDNA was obtained from RNA (5 μg) previously treated with DNAase I with the M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA). Semiquantitative RT-PCR was performed to assess vimentin and E-cadherin expression with the following primers: (forward) 5'-CCACCAAAGTCACGCTGAA-3'and (reverse) 5'-TGCTTGGA TTCCAGAAACG-3' for E-cadherin, and (forward) 5'-AAAGTGT GGCTGCCAAGAAC.-3' and (reverse) 5'-AGCCTCAGAGAG AGGTCAGCAA-3' for vimentin. Hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) was used as an internal standard. Samples carrying the isolated RNA (non-reversed transcribed) or water were used as a control of specificity. EAG1 expression was studied by real-time RT-PCR using the TaqMan™ detection system (Applied Biosystem, Foster City, CA, USA). *HPRT* was used as an internal standard. Analysis from real-time PCR data was performed by the standard curve method. Samples carrying either the isolated RNA (non-reversed transcribed) or water were used as a control for specificity.

Scratch assay. Cell migration was performed as an *in vitro* scratch assay (27). A549 cells were plated in 24-well dishes and maintained in 10% BFS-F12-K until reaching confluence. The cell monolayer was scraped with a p200 pipette tip. Detached cells were removed by washing with PBS until the wounded area was clear then 0.5% BFS-F12-K with or without TGFβ1 was added. Cells were incubated at 37˚C (95% humidity and 5% CO_2) for 48 hours. Plates were observed under a phase-contrast microscope (Olympus DP70; Melville, NY, USA).

Flow cytometry. A549 cells (10^5) were plated in 96-well dishes. Cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD PharMingen, San Diego, CA,USA) according to manufacturer's instructions. A549 cells were incubated for two hours with the specific anti-EAG1 monoclonal antibody, mAb62 (1:50) or the isotype control antibody (1:50), both kindly provided by Walter Stühmer and Luis Pardo (Max-Planck Institute for Experimental Medicine, Göttingen, Germany). Afterwards, cells were incubated with the secondary antibody (1:100) (goat anti-mouse Ig antibody labeled with FITC; DAKO, Carpinteria, CA, USA) for 60 minutes. Data was analyzed in a FACSCalibur (Becton Dickinson, San Jose, CA, USA) with WinMDI 2.9 software.

Statistical analysis. Student's *t*-test was used to compare data between different groups. *P*-values <0.05 or <0.01 were considered to be statistically significant. Analysis was carried out using GraphPad Prism software version 3.0.

Results

In order to induce an EMT process, A549 cells were treated with TGFβ1 (7.3 ng/ml) for 48 hours as previously reported (24, 25). Epithelial cells undergoing EMT change their morphology. As expected, TGFβ1 produced a fibroblast-like morphology in contrast to untreated cells which preserved their epithelial morphology (Figure 1A). Transcriptional down-regulation of E-cadherin and up-regulation of vimentin are hallmarks of the EMT process (28). Following treatment with TGFβ1, A549 cells showed a lower mRNA expresion of E-cadherin and a higher mRNA vimentin expression in comparison to untreated cells (Figure 1B). Wound-healing assay was used to assess cell migration (27). A549 cells undergoing EMT showed an enhanced ability to migrate in this assay (26). Accordingly, we observed that the 'wounded' area was almost completely restored upon TGFβ1 treatment (Figure 1C). Once we confirmed that TGFβ1 induced the EMT process, we investigated *EAG1* gene and protein expression in A549 lung tumor cells undergoing EMT.

Real-time RT-PCR experiments showed that *EAG1* mRNA expression was strongly up-regulated during EMT (Figure 2A). We then investigated by flow cytometry if EAG1 protein expression was also enhanced. An anti-EAG1 monoclonal antibody directed against an area close to the putative pore region was used (8). Figure 2B shows that the mean fluorescence intensity (MFI) of EAG1 in TGFβ1-treated cells clearly increased in comparison to untreated cells remaining in the 'epithelial state' and incubated with either the mAb62 antibody or the isotype control (IgGκ2b).

Discussion

EMT has been proposed as one of the processes by which cancer cells acquire a malignant phenotype (5). Here we show that the *EAG1* mRNA and protein expression are up-regulated in A549 lung cancer cells undergoing EMT induced by TGFβ1. EMT was confirmed by changes in E-cadherin and vimentin gene expression, as well as by a cell migration assay. EAG1 channels have been suggested as

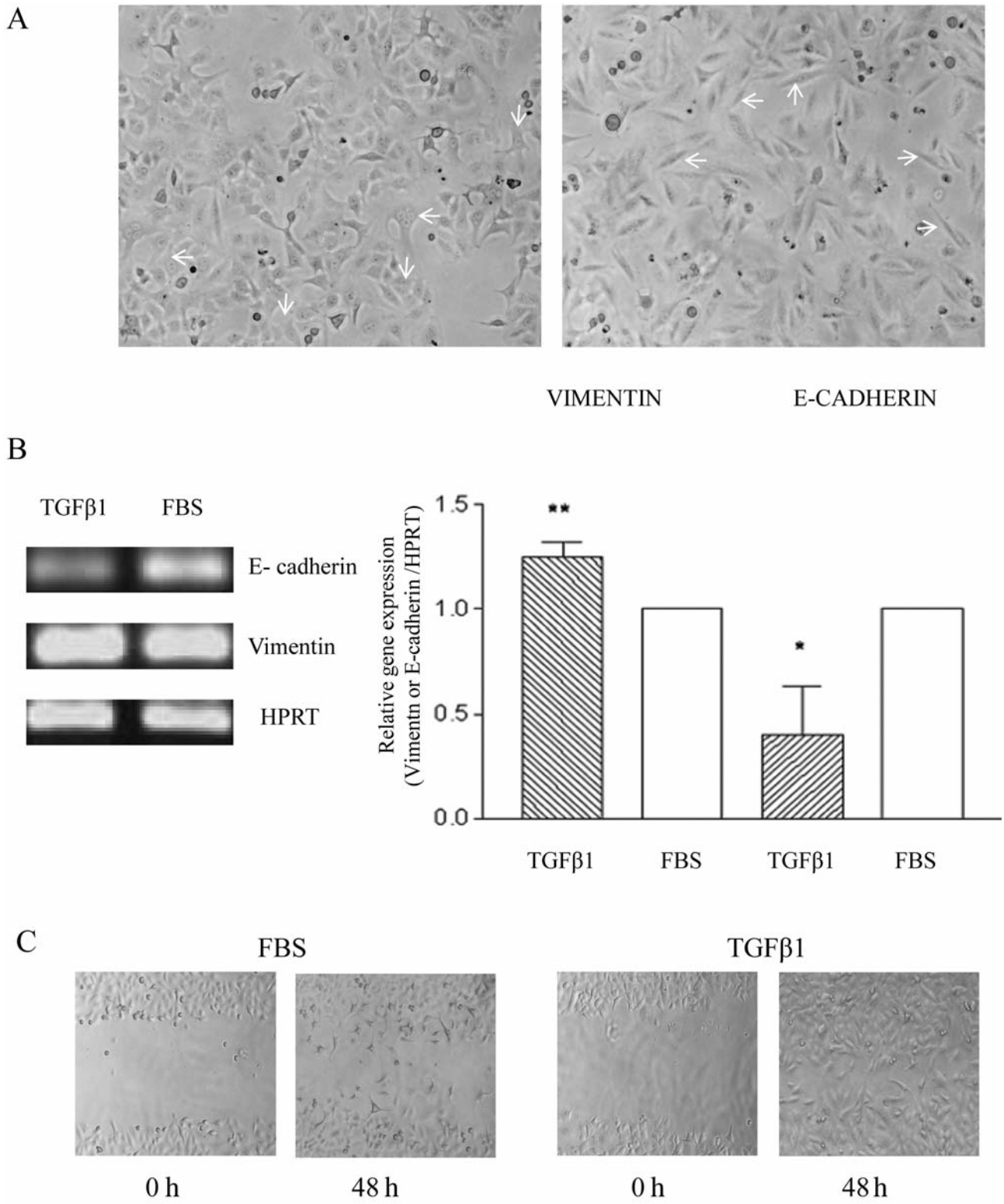

Figure 1. *EMT induction in A549 lung tumor cells. Untreated cells display an epithelial morphology (A, left panel, arrows) but acquire a spindle-shape morphology upon TGFβ1 treatment (right panel, arrows, as expected. B: Gene amplification by RT-PCR of vimentin and E-cadherin (left panel) in A549 cells treated (TGFβ1) or untreated (FBS); right panel shows the relative expression. *p<0.05, **p<0.01 (Columns, mean; bars, SEM; n=5). C: Scratch assay in the presence or absence of TGFβ1 at 0 and 48 hours of treatment. TGFβ1-treated cells display a higher migration (magnification =200×).*

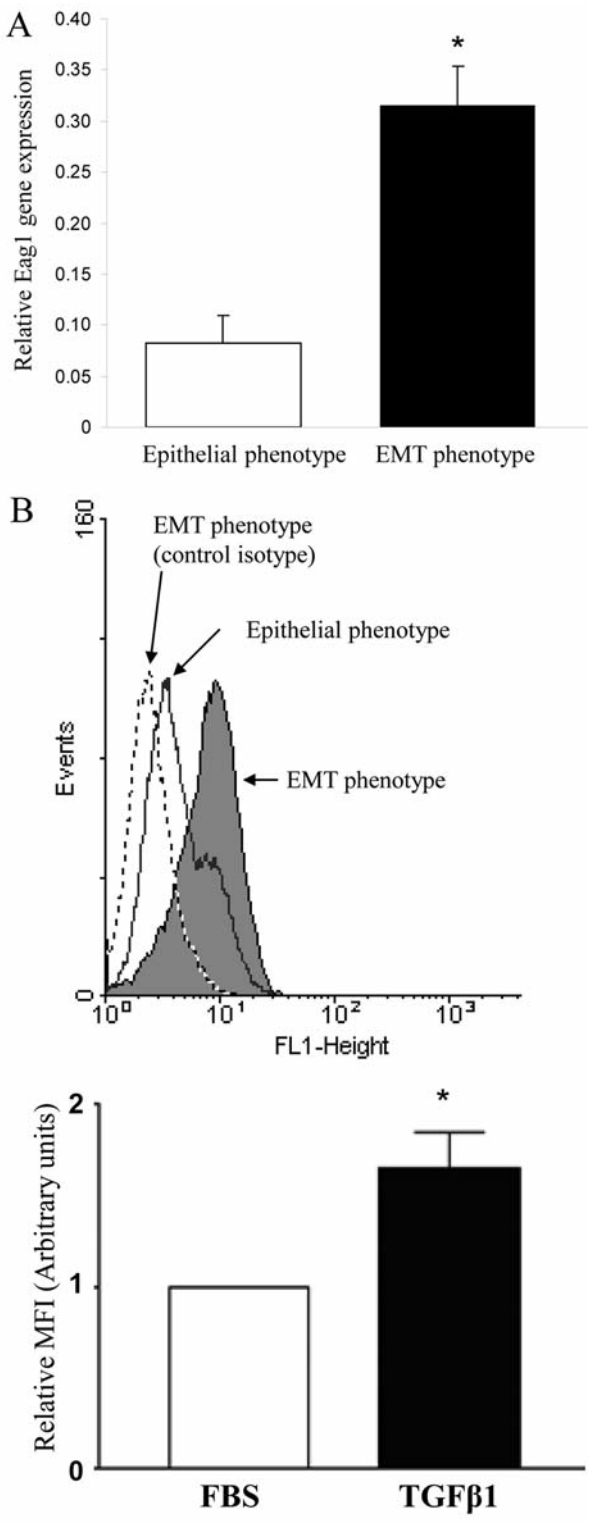

Figure 2. *EAG1 gene and protein expression in EMT. A: Real-time RT-PCR experiments showed a significant increase in EAG1 mRNA expression upon treatment with TGFβ1. B: EAG1 protein expression (assayed by flow cytometry with fluorescent antibodies) was clearly enhanced in the EMT phenotype (left panel); relative expression is shown in the right panel. *p<0.05 (Columns, mean; bars, SEM; n=5).*

potential diagnostic tools and therapeutic targets for many types of cancer (8, 20, 29). In addition, several observations indicate that EAG1 is also involved in tumor cell malignancy. *EAG1* gene amplification has been associated to poor prognosis in colon carcinoma (20) and a higher EAG1 expression has been found in fast growing T84 colon carcinoma cells in comparison to slowly growing carcinoma cells (21). Moreover, EAG1 expression is up-regulated in invasive breast carcinoma (22) and inhibition of its activity decreases migration of leukemia cells (9), and impairs metastasis of lung tumor cells (10). Interestingly, EAG1 is regulated by factors proposed to participate in EMT, namely, IGF-1 (14) and the *E6/E7* oncogenes from HPV-16 (18). Our results suggest that EAG1 also participates in the acquisition of a malignant phenotype in tumor cells.

TGFβ1-induced EMT is a cell cycle-dependent process. AML-12 cells (hepatocyte cell line from mice liver) undergo EMT following treatment with TGFβ only when they are in the G_1/S phase (30). It has been proposed that TGFβ1 provides pre-conditions for epithelial cells to undergo EMT by setting cells in the G_1/S phase (31). Interestingly, EAG1 channel activity is regulated during the cell cycle (32, 33) displaying the highest amplitude during the G_1/S transition in MCF-7 breast cancer cells (34). A feasible scenario is that an increase in the expression of EAG1 is one of the conditions created by TGFβ1 to induce EMT when setting cells in the G_1/S.

EAG1 expression increases the activity of hypoxia-inducible factor 1α (HIF-1α), both under normoxia and hypoxia (12). EMT is induced in both cancerous and healthy cells by HIF-1α (35-37). Moreover, HIF-1α induces the expression of SNAIL and TWIST, which are transcription factors with a pivotal role for EMT (38). Both factors have been identified as poor prognosis markers for non-small cell lung cancer (39). SNAIL is a widely recognized inhibitor of E-cadherin expression and regulates other aspects of EMT including the expression of mesenchymal markers, inhibition of proliferation through suppression of cyclin D proteins, and increased metalloproteinase protein expression among others. TWIST also inhibits E-cadherin expression and is involved in the up-regulation of fibronectin (a mesenchymal marker) and N-cadherin, which is associated with cell migration and invasion (28, 40). In addition, it is known that HIF-1α also up-regulates the expression of TGFβ1 (1). It is thus plausible to hypothesize that TGFβ1, by setting A549 cells in G_1/S, creates the appropriate environment for EMT to occur. In this phase, along with the other genes involved in EMT, *EAG1* expression is up-regulated, which might promote the activity of HIF-1α and, therefore, reinforce the expression of transcription factors involved in EMT and induce the expression of more TGFβ1. Notably, it has been observed that CHO cells bearing EAG1 lose cell-contact inhibition (7) and show an actin-cytoskeleton rearrangement, suggesting an active migration sate (23).

In spite of a non-inactivating outward current recorded in A549 cells undergoing EMT (data not shown), the Cole-Moore shift that characterizes EAG1 currents was not displayed. Actually, recording of endogenous EAG1 channel activity has been either difficult or not possible to achieve in many cell types, including cells from the central nervous system which shows a clear *EAG1* mRNA or protein expression. Astemizole, a non-specific EAG1 inhibitor did not prevent EMT (data not shown), suggesting that the potential role of EAG1 in EMT is not uniquely related to its conducting function. This is in accordance with the observed tumor growth supported by non-conducting EAG1 channels (12). EAG1 has a bipartite nuclear targeting signal in its carboxy terminus (41). Interestingly, a *C*-terminal fragment of a voltage-gated calcium channel translocates to the nucleus and regulates transcription (42). Whether EAG1 plays a similar role in the nucleus, and then in proliferation and EMT, remains to be elucidated. *EAG1*-silencing experiments are needed in order to determine the role of this ion channel during the EMT process.

Our results show that *EAG1* expression is up-regulated when A549 lung tumor cells acquire a malignant phenotype through an EMT process. This might explain why EAG1 is overexpressed in cancer cells with a malignant behavior and why this channel is associated to metastasis and poor prognosis. Our data emphasizes the potential clinical relevance of EAG1 channels in cancer diagnosis and therapy.

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Conflicts of Interest

The Authors have no conflicts of interest.

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