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EGF Receptor Activation Decreases Retroviral Gene Transfer through Protein Kinase C-δ

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Although much progress has been made in the design of retrovirus vectors, the interactions of recombinant retrovirus with host cells remain largely elusive. The inability of recombinant retrovirus to transduce non-dividing cells prompted several studies to determine optimal cocktails of growth factors and/or extracellular matrix molecules to promote gene transfer to slowly diving cells and stem cells. In contrast to previous reports that growth factors increased gene transfer, we found that treatment of human epidermal keratinocytes and several cell lines with epidermal growth factor receptor (EGFR) ligands EGF, transforming growth factor-α, or heparin-binding-EGF decreased gene transfer. Conversely, treatment with an EGFR function-blocking antibody or inhibition of EGFR tyrosine phosphorylation enhanced gene transfer in a dose-dependent manner. In addition, blocking protein kinase C (PKC)-δ but not PKC-ζ, with chemical inhibitors or small interfering RNA reversed the effects of EGF and restored gene transfer, indicating that the effect of EGFR activation is mediated through PKC-δ. Lastly, cell cycle analysis showed that the effect of EGFR activation on retroviral gene transfer was independent of the cell cycle status of target cells. Our results implicate EGFR and PKC-δ in retroviral infection and may have implications for retrovirus gene transfer or design of antiretroviral therapies.

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INTRODUCTION

There are four members in the epidermal growth factor receptor (EGFR) family, namely EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. Upon ligand binding, ErbB family members homo- or hetero-dimerize, *e.g.* ErbB1 and ErbB2, inducing cross-phosphorylation of tyrosine residues and subsequent activation of multiple signaling pathways. Ligands that bind exclusively to EGFR include EGF, transforming growth factor (TGF)-α, amphiregulin, and β-cellulin. Heparin-binding (HB)-EGF and epiregulin can bind EGFR and ErbB4, whereas heregulins bind to ErbB3 and ErbB4 but not to EGFR.[1] ErbB2 is called an "orphan" receptor because no ligand has been identified that binds to its extracellular domain and signal transduction is thought to occur through heterodimerization with other ErbB family members.

Epidermal keratinocytes express EGFR, ErbB2, and ErbB3 but not ErbB4.[2,3] EGFR signaling affects multiple aspects of epidermal cell function, including keratinocyte proliferation, colony formation, differentiation, and survival.[4] Interestingly, the biological effects of EGFR activation depend on the duration of signaling[5] and the state of keratinocyte differentiation.[6] Specifically, activation of EGFR induces proliferation of basal cells but promotes differentiation of suprabasal keratinocytes, regulating the balance between proliferation and differentiation in multilayered epidermis.[6] EGFR and the subsequent mitogen-activated protein kinase pathway can also be activated in the absence of exogenous ligands, through adherens junctions that are assembled at cell–cell contacts during keratinocyte differentiation.[7] On the other hand, inhibition of EGFR promotes assembly of desmosomal junctions increasing cell–cell adhesion in oral squamous carcinoma cells.[8] Therefore, activation of EGFR may have diverse effects on epithelial cells depending on the state of cell differentiation and the local microenvironment.

The protein kinase C (PKC) family of isoenzymes contains at least 10 serine-threonine kinases that are classified into three groups: classical (α, β1, β2, and γ), novel (δ, ε, η, and θ), and atypical (ζ and λ/ι). Phorbol esters activate classical and novel PKCs, calcium activates only classical PKCs, whereas atypical PKCs are activated neither by phorbol esters nor by calcium. In general, PKCs have been implicated in diverse cellular functions, including proliferation, differentiation, and apoptosis through multiple signaling pathways.[9] In particular, PKC-δ has been shown to play a role in the regulation of cell proliferation and programmed cell death with significant implications in immune function and cardiovascular remodeling.[10] Recent studies identified a distinct role of PKC-δ activation involving tyrosine phosphorylation by the Src family kinases.[11,12] In keratinocytes, PKC-δ was shown to play a role in calcium-induced differentiation through a pathway that involved TGF-α, EGFR, Src, and ras^{Ha}.[11,13,14] PKC-δ was also implicated in phosphorylation and translocation of integrin $\alpha6\beta4$ into the cytoplasm and decreased cell attachment to laminin.[15] Despite an explosion of informa-

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tion in the field of PKC signaling, there have been no studies implicating PKC-δ in retroviral infection.

It is well established that recombinant retrovirus infects only actively proliferating cells because retroviral integration occurs only during mitosis[16] and retroviral particles lose their activity after internalization into the cell cytoplasm.[17] To overcome this limitation, several studies employed growth factor cocktails to enhance retroviral transduction by promoting proliferation of target cells. In particular, various combinations of growth factors, *e.g.* stem cell factor, interleukin-3, interleukin-6, thrombopoietin, Flt3, kit-ligand, or extracellular molecules, *e.g.* recombinant fibronectin increased retroviral gene transfer to hematopoietic stem cells.[18–21] Keratinocyte growth factor was also used to enhance gene transfer to lung epithelial cells in three-dimensional cultures as well as following intratracheal administration *in vivo*.[22–24] Follistatin, keratinocyte growth factor, hepatocyte growth factor, or their combinations were successful in enhancing retroviral gene transfer to liver *in vivo*.[25,26] Finally, rFN increased gene transfer to epidermal keratinocytes and in particular to epidermal stem cells.[27,28]

Based on these studies, we hypothesized that treatment with EGFR ligands may promote proliferation and increase gene transfer to human epidermal keratinocytes. Despite previous data supporting this hypothesis, we were surprised to find that EGF, TGF-α, or HB-EGF decreased gene transfer in a dose-dependent manner. In addition, blocking EGFR with a function-blocking antibody or a tyrosine kinase inhibitor restored gene transfer, suggesting that EGFR signaling may reduce retrovirus infection. Use of biochemical inhibitors and small interfering RNA (siRNA) technology identified a novel PKC, namely PKC-δ, as the major player mediating the effect of EGFR activation on retroviral transduction. Our findings may have significant implications in the development of strategies to enhance gene transfer, promote targeting to certain cell types, or design antiretroviral therapies.

RESULTS

EGF decreases retroviral gene transfer

Growth factors such as EGF increase epidermal cell proliferation and migration. As retroviral transduction depends on cell cycle and is higher in proliferating cells, we examined whether EGF could increase retroviral gene transfer to human epidermal keratinocytes. Surprisingly, we found that treatment with EGF after transduction decreased gene transfer to approximately 50% of untreated cells (**Figure 1a**).

Next, we examined whether the time of treatment with EGF was important for gene transfer. To this end, EGF (100 ng/ml) was added at different times post-infection and removed at 24 h post-infection when the medium was replenished. Interestingly, treatment with EGF as late as 10 h post-infection decreased gene transfer, suggesting that EGF may affect a late event in the retroviral life cycle (**Figure 1b**). Notably, short-term treatment (2 h) with EGF at different times post-infection decreased gene transfer to a similar extent as long-term treatment (24 h) (**Figure 1c**).

Similar to primary keratinocytes, EGF decreased gene transfer to human epidermoid carcinoma cells derived from skin (A431), squamous cervical carcinoma cells derived from metastatic site omentum (ME180), rat urinary bladder tumor cells (NBT-II), and human mammary gland adenocarcinoma cells (MDA-MB-468) (**Figure 1d**). Interestingly, each of these cells was affected to a different extent by EGF. Conversely, gene transfer to other cells, *e.g.* another keratinocyte cell line (HaCaT), murine fibroblasts (NIH-3T3), or human kidney epithelial cells (293 T) was not affected by EGF. These results suggest that the effects of EGF on gene transfer are not limited to epidermal keratinocytes and that the response to EGF may vary depending upon the physiological state of target cells.

Activation of Erb1 is necessary for decreased gene transfer

Among the ErbB family of receptors, primary keratinocytes do not have ErbB4.[29,30] As ErbB2 does not have a known ligand, we examined which of the other two ErbB receptors mediated the effect of EGF on retroviral infection. Similar to EGF the other two ligands of ErbB1, TGF-α and HB-EGF, also decreased gene transfer significantly to $\sim$40% of control (**Figure 2a**). In contrast, heregulin-α1, which binds to ErbB3 or an unrelated ligand, insulin-like growth factor-1 had no effect, suggesting that ErbB1 may mediate the decrease in retroviral gene transfer.

To examine whether activation of ErbB1 is necessary for inhibiting retroviral infection, epidermal keratinocytes were infected for 2 h and then treated with EGF (100 ng/ml) in the presence of a monoclonal antibody that blocks ErbB1 (Cetuximab, clone C225). We found that C225 neutralized the effect of EGF in a dose-dependent manner (**Figure 2b**). Similarly, EGFR tyrosine kinase inhibitor, PD153035, blocked the effect of EGF in a dose-dependent manner and restored the level of gene transfer completely at the concentration of 100 nM. Interestingly, PD153035 alone (300 nM) increased gene transfer by $\sim$20%, possibly by blocking the basal activity of EGFR (**Figure 2b**).

PKC inhibitor reverses the effect of EGF on gene transfer

To determine which intracellular pathway(s) may mediate the effect of EGF on gene transfer, we used several pharmacological inhibitors to block known intracellular EGF effectors: Erk1/2 (PD98059, 0.25–10 μM), PLCγ (U73122, 0.25–10 μM), PI3 K (LY294002, 1–10 μM), or IP3-activated ER Ca^{2+} channel (2-APB, 5–40 μM). To this end, keratinocytes were infected with recombinant retrovirus for 2 h and then treated with EGF (100 ng/ml) in the absence or presence of these inhibitors. Blocking these pathways did not reverse the effect of EGF on gene transfer (data not shown).

We also used three different inhibitors of PKC, namely GF109203X, Gö6976, and Gö6983. GF109203X is a general PKC inhibitor, whereas Gö6976 is an inhibitor of classical PKCs, *i.e.* PKC-α and PKC-β.[12] Neither GF109203X nor Gö6976 could reverse the effect of EGF on gene transfer even at high concentrations (**Figure 3**). In fact, high concentrations of Gö6976 decreased gene transfer to a greater extent than EGF. In contrast, Gö6983 reversed the effect of EGF and restored gene transfer to the levels attained in the absence of EGF (**Figure 3**).

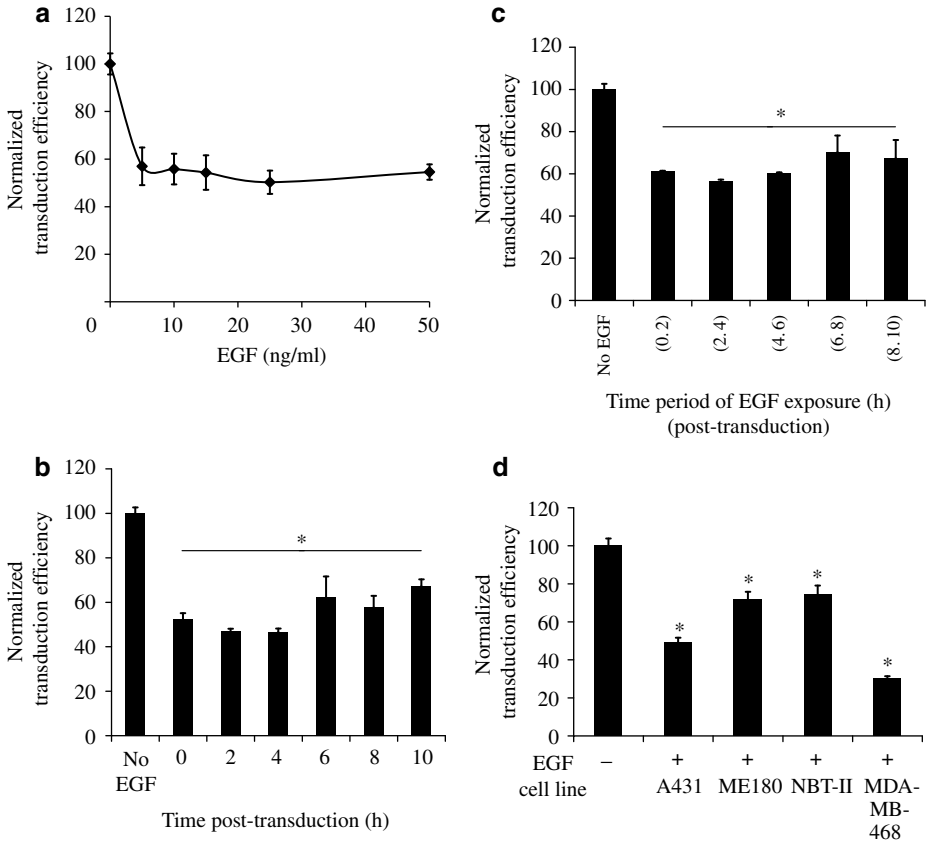

Figure 1 EGF decreased gene transfer to human keratinocytes and several tumor cell lines. Primary epidermal keratinocytes were transduced with GFP-encoding retrovirus for 2 h. (**a**) After transduction, the virus was removed and EGF was added at the indicated concentrations. The next day, the medium was replenished with fresh medium. (**b**) Keratinocytes were transduced for 2 h and at different times after removal of the virus, cells were treated with EGF (100 ng/ml) and 24 h later, the medium was replenished with fresh medium. (**c**) Keratinocytes were transduced for 2 h and at different times after removal of virus, the cells were treated with EGF (100 ng/ml) for a period of 2 h. Then, the medium was replenished with fresh medium. (**d**) The indicated cell lines were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and the cells were treated with EGF (100 ng/ml) for 24 h. The next day, the medium was replenished with fresh medium. (**a**–**d**) Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP+ cells) was normalized to that of cells that were not treated with EGF and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 2$). The asterisks (*) denote $P < 0.05$ between the indicated and control (no EGF) samples.

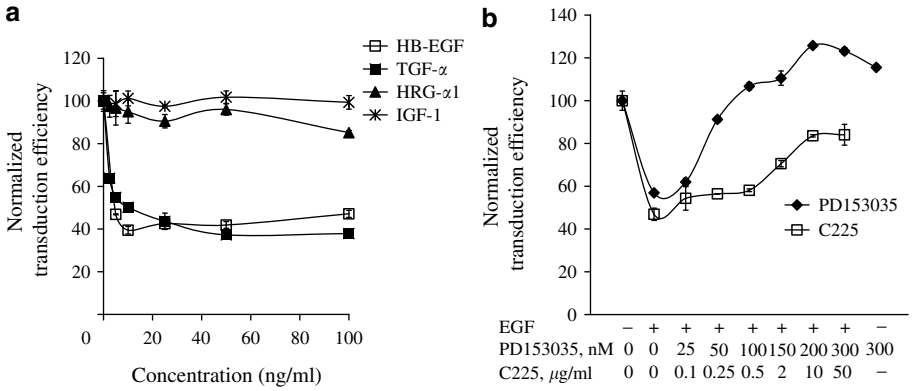

Figure 2 Activation of Erb1 is necessary for decreased gene transfer. Primary epidermal keratinocytes were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and the cells were treated with (**a**) the indicated concentrations of TGF-α, heregulin-α1, HB-EGF, or insulin-like growth factor-1 or (**b**) EGF (100 ng/ml) along with the indicated concentrations of EGFR antibody, C225, or the EGFR tyrosine phosphorylation inhibitor, PD153035. The next day, the medium was replenished with fresh medium. Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP+ cells) was normalized to that of untreated cells and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 3$).

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Similar to primary keratinocytes, a keratinocyte cell line, A431 was also affected by EGFR ligands. Specifically, EGF and TGFα decreased gene transfer by ∼60% and HB-EGF decreased gene transfer by ∼35% (**Figure 4a–c**). In addition, the EGFR antibody, PD153035 and Gö6983, reversed the effects of all three growth factors, restoring gene transfer to its original value (**Figure 4a–c**), although these inhibitors alone did not affect gene transfer significantly (data not shown).

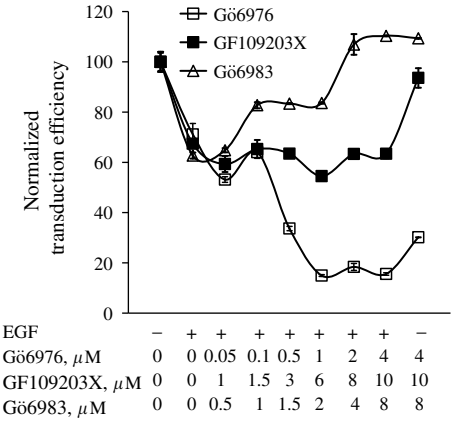

Figure 3 Inhibition of some PKC isoforms reverses the effect of EGF and restores gene transfer. Primary epidermal keratinocytes were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and EGF (100 ng/ml) was added along with the indicated concentrations of PKC inhibitors Gö6976, GF109203X, or Gö6983. The next day, the medium was replenished with fresh medium. Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP+ cells) was normalized to that of untreated cells and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 3$).

PKC-δ mediates the effects of EGF on retroviral infection

Gö6983 is an effective inhibitor of PKC-α, PKC-δ, and PKC-ζ. As the other two inhibitors of PKC-α, GF109203X and Gö6976, did not reverse the effect of EGF, these results suggested that PKC-ζ and/or PKC-δ isoforms may mediate the effect of EGF on retroviral infection. To examine whether PKC-δ or PKC-ζ mediated the effect of EGF and the other ErbB1 ligands on retroviral infection, we employed siRNA to block expression of these two proteins. To this end, we established stable A431 cells lacking PKC-ζ or PKC-δ using siRNA-encoding retroviral vectors and puromycin selection. Expression of siRNA targeted to PKC-ζ-targeted completely abolished the protein (**Figure 5a**), but did not reverse the effect of EGF on the efficiency of gene transfer (**Figure 5b**).

In contrast, reducing the level of PKC-δ reversed the effect of EGF in a dose-dependent manner. Sequential use of one, two, or three siRNAs targeting three different regions of the PKC-δ messenger RNA reduced the protein by 56, 62, or ∼90%, respectively (**Figure 6a** and **b**). Interestingly, the effect of EGF on gene transfer decreased in proportion to the decrease of PKC-δ. Specifically, EGF decreased gene transfer by ∼60% in cells treated with control siRNA but only by ∼15% when PKC-δ

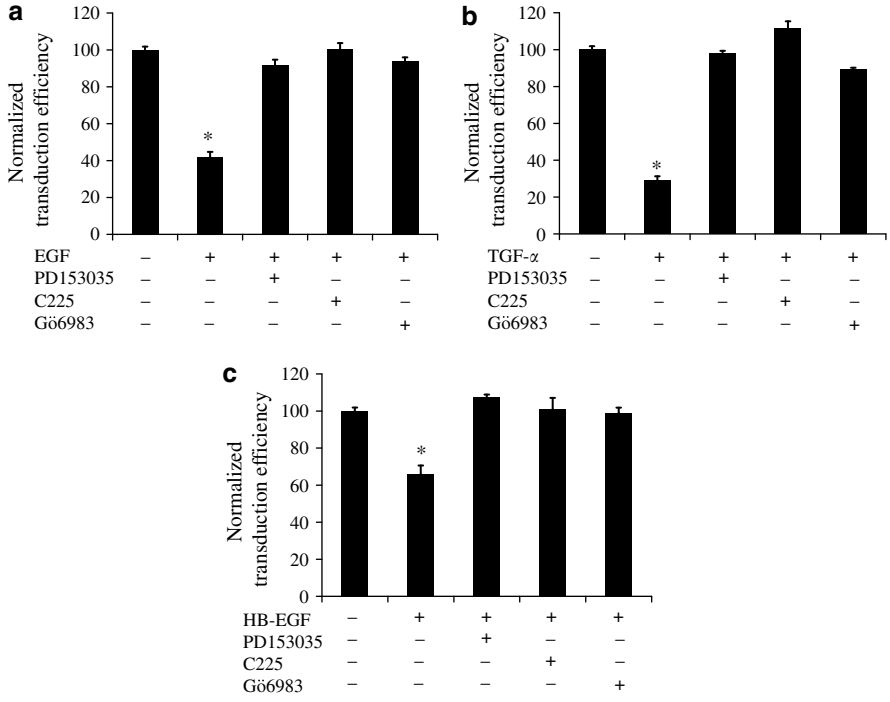

Figure 4 EGFR ligands decreased gene transfer to A431 cells. Epidermal carcinoma A431 cells were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and A431 cells were treated with 100 ng/ml of (**a**) EGF; (**b**) TGF-α; (**c**) HB-EGF in the presence or absence of PD153035 (300 nM), C225 (50 μg/ml) or Gö6983 (8 μM). (**a–c**) Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP+ cells) was normalized to that of untreated cells and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 3$). The asterisks (*) denote $P < 0.05$ between the indicated and control (no growth factor) samples.

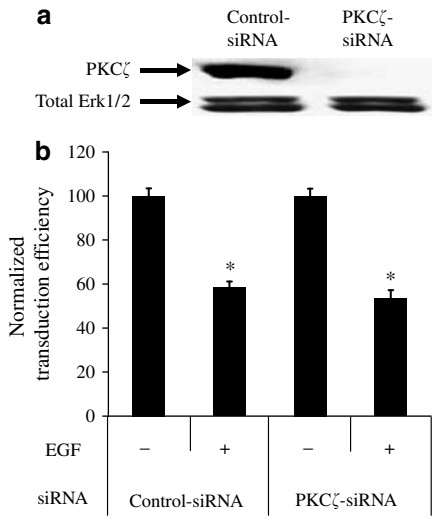

Figure 5 Blocking expression of PKC-ζ does not reverse the effect of EGF on gene transfer. A431 cells were transduced with retrovirus encoding PKC-ζ siRNA or a scrambled control siRNA and selected in puromycin. (**a**) The amount of PKC-ζ was measured using Western blot. (**b**) PKC-ζ-negative and control cells were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and fresh medium without or with EGF (100 ng/ml) was added. Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP + cells) was normalized to that of untreated cells and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 3$). Each asterisk (*) denotes $P < 0.05$ between the indicated and corresponding control (no EGF) sample.

was reduced by ∼90% (**Figure 6c**), suggesting that PKC-δ is necessary to mediate the effects of EGF on retroviral infection.

PKC-δ is phosphorylated at Tyr311 in response to EGF

Next, we examined whether treatment with EGF phosphorylates PKC-δ. We found that phosphorylation of Thr505 and Ser643/676 did not change in response to EGF. In contrast, Tyr311 was phosphorylated as early as 2 min after addition of EGF or TGF-α (**Figure 7**). Interestingly, TGF-α induced more pronounced phosphorylation of Tyr311 than EGF in agreement with the gene transfer data.

The effect of EGFR/PKC-δ on gene transfer is independent of retrovirus envelope

As several viruses have been shown to bind to growth factor or cytokine receptors, it was possible that retrovirus bound to EGFR. If that were the case, addition of EGF during infection would compete with the virus for binding to the receptor decreasing the efficiency of gene transfer. To test this hypothesis, we transduced the cells in the presence of high concentration of the EGFR antibody, C225. We found that gene transfer remained unaffected (**Figure 8a**), suggesting that retrovirus does not bind to EGFR and therefore, EGF does not decrease gene transfer by preventing virus binding to the cell surface.

Furthermore, we employed retrovirus pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) to examine whether the effects of EGF depended on the retroviral envelope. EGF decreased gene transfer by the VSV-G pseudotyped virus

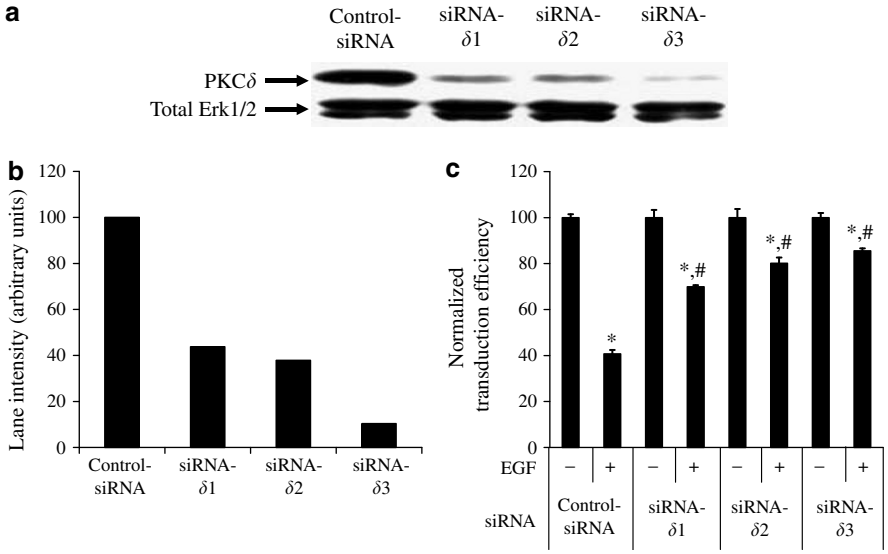

Figure 6 Blocking expression of PKC-δ reverses the effect of EGF and restores gene transfer. A431 cells were subjected to three sequential retroviral transductions to express one (siRNA-δ1), two (siRNA-δ2), or three (siRNA-δ3) siRNAs, each targeting a different region of the PKC-δ messenger RNA. Cells expressing a scrambled siRNA served as negative control. (**a**) PKC-δ siRNA-expressing cells were expanded and the amount of PKC-δ was measured using Western blot. (**b**) Quantitation of Western blot by densitometry. Lane intensity was determined using Kodak software and normalized by loading control. (**c**) siRNA-expressing or control cells were transduced with GFP-encoding retrovirus for 2 h. After transduction, the virus was removed and fresh medium without or with EGF (100 ng/ml) was added. Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP + cells) was normalized to that of untreated cells and plotted as mean ± SD of triplicate samples in a representative experiment ($n = 3$). Each asterisk (*) denotes $P < 0.05$ between the indicated and corresponding control (no EGF) sample. Each pound (#) denotes $P < 0.05$ between the indicated and EGF-treated control siRNA sample.

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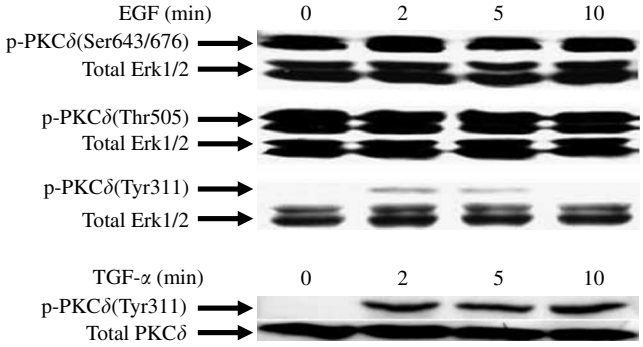

Figure 7 EGF and TGF-α phosphorylate tyrosine 311 of PKC-δ. A431 cells were treated with EGF (100 ng/ml) for the indicated times and phosphorylated PKC-δ at Ser643/676, Thr505, or Tyr311 were measured using Western blot. Tyr311 phosphorylation was also measured after treatment with TGF-α (100ng/ml). Total Erk1/2 or total PKCδ served as loading control ($n=2$).

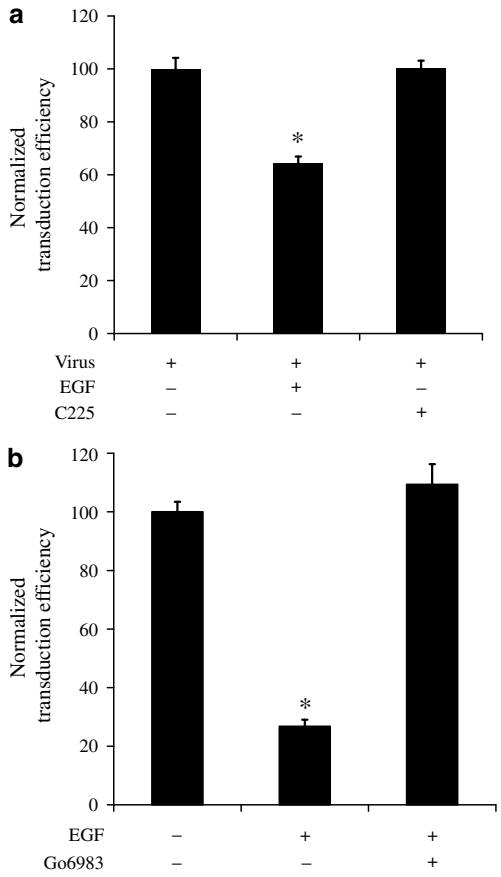

Figure 8 EGFR/PKC-δ decrease gene transfer independent of retroviral envelope. (**a**) Keratinocytes were transduced for 2 h in the presence of EGF (100 ng/ml) or an EGFR-blocking antibody, C225 (50 μg/ml). Then, the virus was removed and fresh medium without EGF or C225 was added. (**b**) A431 cells were transduced with VSV-G-pseudotyped retrovirus for 2 h. After transduction, the virus was removed and cells were treated with 100 ng/ml of EGF in the presence or absence of Gö6983 (8 μM) for 24 h. (**a**, **b**) Flow cytometry was performed when the cells reached 90–95% confluence (3 days post-transduction). The transduction efficiency (% GFP+ cells) was normalized to that of untreated cells and plotted as mean $\pm$ SD of triplicate samples in a representative experiment ($n=2$). The asterisks (*) denote $P<0.05$ between the indicated and control (no growth factor) samples.

(**Figure 8b**) to a similar extent as virus carrying the wild-type amphotropic envelope. In addition, Gö6983 reversed the effect of EGF, suggesting that the effect of EGFR/PKC-δ pathway on gene transfer is independent of the receptor that the virus engages on the cell surface.

DISCUSSION

It is well known that retrovirus infects only dividing cells and infection is cell-cycle dependent mainly because retroviral DNA integration occurs only during mitosis.[16,17,31] As EGF and other EGFR ligands such as TGF-α and HB-EGF are known to stimulate keratinocyte proliferation and decrease expression of epidermal differentiation markers such as keratin-10, transglutaminase, and fillagrin,[32,33] we expected that they would also increase retroviral gene transfer to these cells. Surprisingly, we found that EGF, TGF-α, or HB-EGF decreased the efficiency of gene transfer, whereas blocking EGFR with a monoclonal antibody or inhibiting its tyrosine kinase activity abolished this effect. In addition, Gö6983 and siRNA inhibition of PKC-δ also reversed the effect of EGF, TGF-α, and HB-EGF, suggesting that activation of EGFR decreased gene transfer through PKC-δ.

Our results show that EGFR but not ErbB3 mediates the effects of EGF, TGF-α, or HB-EGF on retroviral infection. EGFR is responsible for the bulk of the activity of EGF family members in keratinocytes, increasing epithelial cell proliferation *in vitro*[32] and promoting development of epithelial tissues such as epidermis, lung, and gastrointestinal tract *in vivo*.[34–36] In addition to promoting growth and migration of basal keratinocytes, activation of EGFR has been shown to promote differentiation of suprabasal cells that are committed to terminal differentiation.[6] The diverse activities of EGF on epidermal keratinocytes have been attributed at least in part to sustained Erk1/2 signaling upon EGFR activation.[5] The distinct effects of EGF on keratinocytes at different stages of differentiation may suggest that EGF and the other EGFR ligands may affect gene transfer to a subpopulation of keratinocytes.

Our results also showed that activation of EGFR induced tyrosine phosphorylation of PKC-δ and that PKC-δ was necessary to mediate the effects of EGFR ligands on retroviral gene transfer. Specifically, chemical inhibition by Gö6983 and siRNA for PKC-δ but not PKC-ζ reversed the effects of EGFR ligands and restored the efficiency of gene transfer in a dose-dependent way. Previous studies showed that TGF-α and v-ras^{Ha} induced tyrosine phosphorylation of PKC-δ possibly through a member of the Src kinase family.[14] Interestingly, calcium-induced differentiation increased the level of TGF-α by 30-fold with a concomitant increase in PKC-δ tyrosine phosphorylation, implicating EGFR in keratinocyte differentiation via a pathway that involves PKC-δ.[37] On the other hand, overexpression of PKC-δ in normal or neoplastic keratinocytes initiated apoptosis[38] and tyrosine phosphorylation of PKC-δ was associated with induction of apoptosis in response to oxidative stress.[39–41] As retroviral infection may also be perceived as stress to the host, tyrosine phosphorylation of PKC-δ by EGFR ligands may cause the cells under stress to activate apoptotic pathways, ultimately decreasing infectivity.

Surprisingly, a short 2-h treatment with EGF had similar effect on gene transfer as 24-h treatment. Previously, it was shown that Erk1/2 phosphorylation in response to EGF or hepatocyte growth factor was sustained for several hours, whereas signaling from keratinocyte growth factor or insulin-like growth factor-1 was transient. This difference in the duration of signaling was responsible for expression of matrix metalloproteinase-9, which in turn caused colony scattering and increased keratinocyte migration.[5] Interestingly, PKC-δ was implicated in a feedback loop that reduced transfer of EGFR from early to late endosomes, decreasing lysosomal degradation and increasing recycling to the cell surface.[42] Therefore, it is possible that activation of PKC-δ by EGFR initiated a feedback loop causing persistent activation of the pathway(s) that ultimately suppressed retrovirus infection. Moreover, treatment with EGF immediately or as late as 10 h after infection reduced gene transfer to a similar extent, suggesting that EGFR activation may affect a late event in the retrovirus life cycle.

Our data showed that reduction of gene transfer by EGFR ligands was more pronounced in A431 and MDA-MB-468 cells than primary keratinocytes, NBT-II, or ME180 cells, whereas gene transfer to another keratinocyte cell line, HaCaT, remained unaffected. The disparate response may be due to either the formation of different receptor heterodimers, *e.g.* ErbB1-ErbB1 vs ErbB1-ErbB2 in different cells or differential expression of the receptors on the cell surface or differential recruitment of adaptor protein(s) that may link EGFR to PKC-δ. Previous studies reported that primary cells expressed different isoforms of ErbB receptors than cell lines and tumor cells, affecting the balance between growth and differentiation.[30] In addition, ras^{Ha}, c-Src, and c-Fyn have been implicated in phosphorylation of PKC-δ upon EGFR activation.[11,13,14,37] The intracellular effectors that are involved in transmission of EGFR signaling may depend on the context of target cells, *e.g.* normal vs tumor cells and the local microenvironment, *e.g.* presence of cytokines or growth factors. Therefore, identifying the mechanism through which EGFR suppresses retroviral infection may help to develop strategies for targeted gene delivery by activating or inhibiting intracellular signaling pathways via growth factors or biochemical inhibitors.

Although the exact mechanism through which EGFR affects retroviral transduction remains unknown, some alternatives can be safely excluded. First, the effect of EGFR and PKC-δ is independent of the virus envelope glycoprotein as EGF decreased gene transfer by retrovirus carrying the wild-type envelope or VSV-G and Gö6983 restored gene transfer in both cases. Also, EGFR is unlikely to be a co-receptor for retrovirus as antibody blocking of EGFR during transduction did not affect gene transfer. Finally, if EGFR were a co-receptor, replacing the wild-type retroviral envelope, gp70 with VSV-G would most likely eliminate or reduce the effect of EGF. Second, our experiments showed that there was no correlation between the effect of EGF on cell proliferation and gene transfer (see only **Supplementary material**, **Supplementary Figure S1** and **Table S1**), suggesting that EGF does not decrease gene transfer by altering the cell cycle status of target cells. Collectively, these data suggest that EGFR ligands do not affect retrovirus binding to the cell surface or entry into the nucleus.

In summary, we implicated EGFR as a negative effector of retroviral infection. We also discovered that the action of EGFR was mediated by PKC-δ, a novel PKC with diverse cellular functions. Our findings add to our understanding of retrovirus–cell interactions and may have significant implications in the development of strategies to enhance gene transfer, improve targeted gene delivery, or design novel antiretroviral therapies.

MATERIALS AND METHODS

Cell culture. Primary human keratinocytes were isolated from neonatal foreskins following the protocol of Green *et al.*[43] and expanded on feeder layers of mitomycin-C treated 3T3-J2 mouse fibroblasts (ATCC, Manassas, VA) as described previously.[44] After the initial expansion, primary keratinocytes were cultured in keratinocyte serum-free medium supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml EGF (Invitrogen, Carlsbad, CA). HaCaT, ME180 cells (kindly provided by Dr Satrajit Sinha, Department of Biochemistry, University at Buffalo, SUNY), and A431 carcinoma cells, rat urinary bladder tumor cells (NBT-II), human mammary gland adenocarcinoma cells (MDA-MB-468), and human embryonic kidney cell-derived retrovirus-packaging cell line HEK 293T/17 (ATCC) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% certified fetal bovine serum (Gibco BRL, Grand Island, NY), 100 U of penicillin, and 100 μg/ml streptomycin (Gibco BRL). Retrovirus-producing PA317 cells (kindly provided by Dr Steven J Greenberg, Roswell Park Cancer Institute, Buffalo, NY[45]) were cultured in Dulbecco's modified Eagle's medium containing 10% defined fetal bovine serum (Hyclone, Logan, UT), 100 U of penicillin, and 100 μg/ml streptomycin.

Retrovirus production. The LXCGN retroviral vector was derived from the parent vector LXSN, originally described by Miller and Rosman.[46] LXCGN encodes for enhanced green fluorescence protein (GFP)-neomycin phosphotransferase fusion protein (EGFP-Neo^r) transcriptionally controlled by the cytomegalovirus-derived promoter.[45] GFP-encoding amphotropic retrovirus was produced from PA317 cells as described previously.[47]

VSV-G pseudotyped GFP-encoding retrovirus was produced by transient transfection of HEK 293T/17 cells with the retroviral vector BMN-I-GFP (kindly provided by Dr Gary Nolan, Stanford University, Stanford, CA) and the VSV-G encoding plasmid. Briefly, HEK 293T/17 cells were plated in T-75 tissue culture flask (5×10^6 per flask) and incubated overnight. The next day, retroviral plasmids (18.5 μg of BMN-I-GFP and 1.5 μg of VSV-G) and transfection reagent Fugene 6 (Roche, Indianapolis, IN) were mixed (1:3, μg:μl) in 800 μl of Dulbecco's modified Eagle's medium without serum or antibiotics. The mixture was incubated at room temperature for 25 min and then added to the culture medium overlaying the packaging cells. The next day ($\sim$18 h later), the medium was replaced with fresh culture medium and retrovirus was harvested 24 h later.

Retrovirus transduction. Keratinocytes were plated on 24-well tissue culture plastic plates (Discovery Labware, Bedford, MA) at 2×10^4 cells/cm^2 in serum-free medium containing bovine pituitary extract but no EGF (primary human keratinocytes) or Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HaCaT, A431, ME180, NBT-II, MDA-MB-468). The next day, cells were infected with retroviral supernatant containing 8 μg/ml polybrene for 2 h. Then, the virus was removed and fresh medium containing various concentrations of the indicated growth factor was added for 24 h. Different concentrations of the following growth factors were used: EGF, HB-EGF, TGF-α (EMD

Biosciences, San Diego, CA), heregulin-α1 (Lab Vision, Fremont, CA), and insulin-like growth factor-1 (R&D systems, Minneapolis, MN). Following the 24-h treatment, growth factors were removed and replaced by fresh culture medium.

In some experiments, cells were treated with growth factors in the presence of pharmacological inhibitors to block different transduction pathways. EGFR inhibitor (PD153035; EMD Biosciences) and PKC inhibitors GF109203X, Gö6976, and Gö6983 (EMD Biosciences) were used at the indicated concentrations and vehicle dimethylsulfoxide was used as control. EGFR-blocking antibody, Cetuximab (C225), was a generous gift from ImClone Systems Incorporated (New York, NY). Inhibitors of Erk1/2 (PD98059), PI3 K (LY294002), PLCγ (U73122), and IP3-induced Ca^{2+} channel blocker (2-APB) were all from EMD Biosciences.

Western blotting. Keratinocytes were plated in six-well plates (1.5×10^6 cells/well) and 24 h later, cells were treated with different growth factors as indicated in each experiment. At different times, the cells were lysed using ice-cold lysis buffer: 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) sodium dodecyl sulfate, 10% glycerol, and 0.01 (w/v) bromophenol blue (Cell Signaling, Danvers, MA) containing 41.67 mM dithiothreitol (Cell Signaling) and protease inhibitor cocktail (Complete Mini; Roche Applied Science, Indianapolis, IN). Lysates were frozen at −80°C until use.

For gel electrophoresis, the samples were sonicated five times for 5 s per treatment, then heated at 95°C for 5 min, placed on ice for 10 min, centrifuged for 5 min at 10 000 × *g*, loaded onto a 8–10% denaturing gel (sodium dodecyl sulfate—polyacrylamide gel electrophoresis), and run for 60–90 min at 150 V (Mini-protean 3 system; BioRad Laboratories, Hercules, CA). The proteins were then transferred onto a polyvinylidene difluoride membrane (Immun-Blot™ polyvinylidene difluoride, BioRad Laboratories) for 1 h at 350 mA using an electrophoretic transfer cell (Mini Trans-Blot®; BioRad Laboratories). The membrane was incubated in blocking agent (5% (w/v) non-fat milk in wash buffer (Tris-buffered saline/0.1% Tween-20)) for 1 h at room temperature. The membrane was incubated with rabbit anti-human polyclonal antibody (PKC-δ, PKC-ζ, phospho-PKC-δ (S643/676, T505, Y311); 1:1,000 dilution in blocking agent; overnight at 4°C; Cell Signaling). The membrane was washed five times with wash buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G polyclonal secondary antibody (1:2,000 dilution in blocking agent for 1 h at room temperature; Cell Signaling). The membrane was washed five times with wash buffer and the bands were detected using chemiluminescence (LumiGLO; KPL, Gaithersburg, MD) according to the manufacturer's instructions. Luminescence intensity was quantified using Kodak 1D v.3.6.1 software (Kodak Scientific Imaging Systems, New Haven, CT). The membranes were stripped and re-probed with anti-Erk1/2 or anti-PKC-δ (1:1,000 dilution in blocking agent; overnight at 4°C; Cell Signaling) as loading control.

siRNA inhibition of PKC-δ and PKC-ζ. siRNA-encoding retrovirus was used to block expression of PKC-ζ or PKC-δ in A431 cells. Oligonucleotide encoding for PKC-ζ siRNA in pRetro-SUPER retroviral expression vector (Oligoengine, Seattle, WA) was kindly provided by Dr Debabrata Mukhopadhyay (Department of Biochemistry and Molecular Biology and Cancer Center, Rochester, Minnesota).[48] Three oligonucleotides encoding siRNAs targeting different regions of the *pkc-δ* messenger RNA were designed and cloned between *Bam*H1 and *Eco*R1 sites of pSIREN-RetroQ vector according to the manufacturer's instructions (Clontech, Mountain View, CA). The sequences were as follows: PKC-δ-siRNA1 (5′-GATCCGAAGCCGACCATGTATCCTTTCA AGAGAAGGATACATGGTCGGCTTCTTTTTTGCTAGCG-3′); PKC-δ-siRNA2 (5′- GATCCGATGAAGGAGGCGCTCAGCTTCAAGAGAGCTG AGCGCCTCCTTCATCTTTTTTGCTAGCG-3′); and PKC-δ-siRNA3 (5′- GATCCGGCTGAGTTCTGGCTGGACTTCAAGAGAGTCCAGCCA GAACTCAGCCTTTTTTGCTAGCG-3′). An oligonucleotide generating a non-silencing control siRNA (5′- GATCCGAATTCTCCGAACGTGT CACGTTCAAGAGAACGTGACACGTTCGGAGAATTCTTTTTTAGTAC TG-3′)[49] was also cloned in pSIREN-RetroQ vector and used as control.

A431 epidermoid carcinoma cells were transduced with retrovirus encoding for PKC-δ-siRNA1 or control siRNA. After selection with puromycin (0.4 μg/ml), A431 cells were subjected to one or two additional sequential transductions to express PKC-δ-siRNA2 and PKC-δ-siRNA3. A431 cells expressing one, two, or three PKC-δ siRNAs were denoted as siRNA-δ1, siRNA-δ2, or siRNA-δ3, respectively. The level of PKC-δ and PKC-ζ proteins in transduced A431 cells was detected by Western blot.

Flow cytometry. Gene transfer efficiency was measured as the fraction of GFP+ cells using flow cytometry 3 days after transduction as described previously.[27,28,50]

Statistical analysis. Statistical analysis of the data was performed using a two-tailed Student's *t*-test using Microsoft Excel (Microsoft, Redwood, CA). The data were considered statistically different when $P < 0.05$.

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SUPPLEMENTARY MATERIAL

Materials and Methods, Results.
Figure S1. Effect of EGF on cell proliferation.
Table S1. Effect of EGF on cell cycle.

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