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Autocrine epidermal growth factor signaling stimulates directionally persistent mammary epithelial cell migration

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Cell responses to soluble regulatory factors may be strongly influenced by the mode of presentation of the factor, as in matrix-bound versus diffusible modes. The possibly diverse effect of presenting a growth factor in autocrine as opposed to exogenous (or paracrine) mode is an especially important issue in cell biology. We demonstrate here that migration behavior of human mammary epithelial cells in response to stimulation by epidermal growth factor (EGF) is qualitatively different for EGF presented in exogenous (paracrine), autocrine, and intracrine modes. When EGF is added as an exogenous factor to the medium of cells that express EGF receptor (EGFR) but not EGF, cell migration speed increases while directional persistence decreases. When these EGFR-expressing cells are made to also express via retroviral transfection EGF in protease-cleaveable transmembrane form on the plasma membrane, migration speed similarly increases, but directional persistence increases as well. Addition of exogenous EGF to these cells abrogates their enhanced directional persistence, reducing their directionality to a level similar to wild-type cells. If the EGFR-expressing cells are instead transduced with a gene encoding EGF in a soluble form, migration speed and directional persistence were unaffected. Thus, autocrine presentation of EGF at the plasma membrane in a protease-cleavable form provides these cells with an enhanced ability to migrate persistently in a given direction, consistent with their increased capability for organizing into gland-like structures. In contrast, an exogenous/paracrine mode of EGF presentation generates a "scattering" response by the cells. These findings emphasize the functional importance of spatial restriction of EGFR signaling, and suggest critical implications for growth factor–based therapeutic treatments.

Introduction

Autocrine receptor/ligand signaling loops were first identified in tumor cells, where it was found that transformation of cells resulted in overexpression of certain growth factors leading to unregulated proliferation of the tumor cells (Sporn and Todaro, 1980). However, in the ensuing decades autocrine signaling has been found to operate in numerous physiological situations (Sporn and Roberts, 1992), including wound healing (Tokumaru et al., 2000), angiogenesis (Seghezzi et al., 1998), and tissue organization during development (Wasserman and Freeman, 1998) and reproductive cycles (Xie et al., 1997). Although it is becoming evident that autocrine loops play crucial roles in regulation of cell function within tissue contexts, it is unclear whether their effects on cell responses are different from the effects of the same ligand presented in exogenous or paracrine manner. Hypotheses that the effects of autocrine presentation may be significantly disparate from those of exogenous/paracrine presentation can be motivated, at least, by analogous findings that cell responses to some ligands are different depending on whether the ligands are presented in soluble versus substratum-bound form (Massague and Pandiella, 1993). An underlying mechanistic foundation for this concept is provided by the recently growing evidence that diversity of pathway activation from a given receptor is dependent on compartmental location of the signaling ligand–receptor complexes (Carraway and Carraway, 1995; DiFiore and Gill, 1999; Ceresa and Schmid, 2000).

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A particular and important case in point is the organization of mammary epithelial cells (MECs)* into structured tissues regulated by EGF receptor (EGFR)-mediated autocrine signaling by various epidermal growth factor (EGF) family ligands (Wiley et al., 1998). EGF family ligands, including EGF itself, transforming EGF-like growth factor α (TGFα), amphiregulin (AR), and heparin-binding growth factor (HB-EGF), are normally synthesized as transmembrane precursors which are then proteolytically cleaved and processed to release the mature peptide ligand (Massague and Pandiella, 1993). The various EGF family ligands show diverse levels of expression among cell types and tissue context, and the issue of whether each might serve distinct physiological roles is under vigorous investigation (Cook et al., 1995; Dempsey et al., 1997; Sweeney and Carraway, 2000).

To test the hypothesis that autocrine presentation of EGF family ligands provides for a distinct manner of cell regulation, we have generated an experimental cell system in which the presentation mode of EGF in a MEC line can be altered. Wiley et al. (1998) used retroviral transfection to individually express each of two forms of EGF ligands in the EGFR-expressing human MEC (HMEC) line (Stampfer and Yaswen, 1993) (Fig. 1). One is the mature EGF 53 amino acid peptide retaining its cytoplasmic and transmembrane domain (EGF-Ct); this version is protealytically cleaved at the cell plasma membrane, diffuses into the extracellular environment and is recaptured by cell-surface receptors, thus operating in classical autocrine mode. The second is simply the mature EGF 53 amino acid peptide containing a signal sequence (sEGF). This version is generated in soluble form in the lumen of the protein synthesis pathway where it can bind newly synthesized (and perhaps recycling) intracellular EGFR, thus operating in a presumably pathological intracrine mode. The parental HMEC cells (subtype 184A1) produce low levels of endogenous EGFR ligands and thus require exogenous EGF for optimum growth in culture. This allows them to be used to compare autocrine and intracrine presentation modes of EGF with that of typical paracrine mode, where the ligand is provided exogenously in the cell culture medium.

The disparate effects of these three modes of EGF presentation on HMEC organization in vitro were striking (Wiley et al., 1998). Presentation in the intracrine (sEGF) mode resulted in a signaling loop that stimulated cell proliferation, but in a structurally disorganized fashion. This loop was uninterruptable, as a monoclonal antibody that blocks EGF binding to EGFR was unable to inhibit the proliferation response. In contrast, presentation in the autocrine (EGF-Ct) mode stimulated HMEC to not only proliferate but also to migrate into organized units resembling gland structures with lobular and ductal aspects. Both the proliferation and organizational responses of the cells to EGF-Ct were inhibited by the anti-EGFR blocking antibody. Moreover, and perhaps even most surprisingly, addition of exogenous EGF inhibited the ability of the EGF-Ct–expressing cells to organize. These findings indicate that autocrine presentation of EGF provides a migration-related signal involved in structural organization that is not provided by either intracrine or exogenous/paracrine presentation.

Thus, we were motivated for this present study to directly test whether autocrine presentation of EGF stimulates a migration behavioral response in HMEC qualitatively different from that stimulated by intracrine and exogenous/paracrine presentation of the same ligand. Indeed, we find that autocrine (EGF-Ct) presentation not only stimulates rapid HMEC migration, but also increases directional persistence. In contrast, intracrine (sEGF) and exogenous/paracrine EGF stimulates migration behavior characterized by low directional persistence, similar to the "scattering" behavior displayed by a variety of different cell types given exogenous growth factors (Warn, 1994; Stella and Comoglio, 1999). Significantly, the highly persistent migration of the HMEC-expressing EGF-Ct is lost when the autocrine EGF–EGFR signaling is inhibited by addition of the anti-EGFR–blocking antibody or is supplemented by addition of exogenous EGF. This suggests that autocrine presentation of EGF yields a spatial localization of EGF–EGFR-signaling complexes that drive the persistent locomotion behavior. This may be part of a general mechanism involved in tissue organization and remodeling.

Results and discussion

MEC migration speed is increased by signaling from cell surface EGF–EGFR complexes

Wild-type (WT) HMEC as well as cells expressing either sEGF or EGF-Ct were used in these studies. The structure of the artificial sEGF and EGF-Ct genes are shown in Fig. 1. Migration speeds of HMEC for the three different EGF presentation modes were quantified using time-lapse video microscopy of individual cells (Fig. 2). In the absence of exogenous EGF, WT HMEC exhibited a baseline mean migration speed of ~50 μm/h. EGF-Ct–expressing cells (autocrine presentation) exhibited substantially greater mean speed at ~120 μm/h, whereas sEGF-expressing cells (intracrine presentation) showed only a small increase in mean speed to roughly 65 μm/h. Addition of 2 nM exogenous EGF increased the mean migration speeds of both WT and sEGF-expressing cells to 90 μm/h, whereas the mean speeds of EGF-Ct–expressing cells remained at 120 μm/h. The differences among mean speeds of the three cell types in the presence of 2 nM exogenous EGF are not statistically significant. Addition of 225 mAb, which blocks the binding of extracellular, but not intracellular EGF to its receptor (Wiley et al., 1998), decreased the mean migration speed of each of the cell types to the range of 25–35 μm/h. The reduction from the original WT and sEGF cell baselines of 50–65 μm/h is likely due to blocking of the low levels of endogenous EGF family autocrine ligands in these cells (Wiley et al., 1998). Together, these findings indicate that the migration speed of the HMEC is essentially governed by signaling from cell surface EGF–EGFR complexes; this is consistent with recent evidence that key pathways of EGFR-mediated induction of enhanced cell migration reside at the plasma membrane (Haugh et al., 1999; Glading et al., 2001). Signaling by EGF–EGFR complexes at the cell surface in EGF-Ct–expressing HMEC is relatively unaffected by additional

*Abbreviations used in this paper: EGFR, EGF receptor; HMEC, human MEC; MEC, mammary epithelial cell; WT, wild-type.

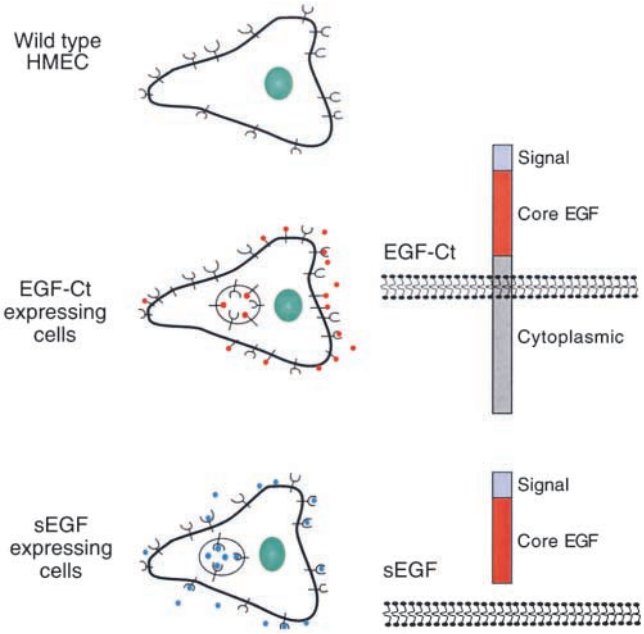

Figure 1. **Cell types used in this study.** WT HMECs express EGFR endogenously, but lack the expression of EGF. EGF-Ct cells express the EGF-Ct construct. EGF-Ct lacks the NH_2-terminal extracellular domain of the human EGF precursor. The sEGF construct is simply the 53 amino acid long mature EGF without the NH_2-terminal, COOH-terminal, or the transmembrane domain of EGF precursor; it binds to the EGFR during receptor transport to the cell surface (Wiley et al., 1998).

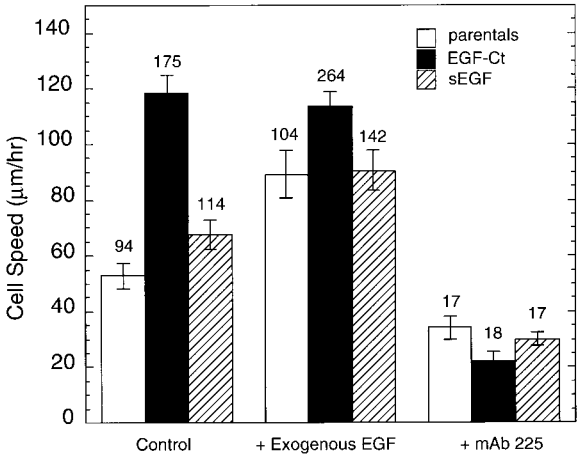

Figure 2. **Effect of ligand presentation on cell speed.** Filled, hatched, and blank bars represent the cell speeds of EGF-Ct–expressing cells, sEGF-expressing cells, and WT HMECs, respectively. Cell speeds of the three cell types were monitored in the absence of exogenous EGF, in the presence of 2 nM exogenously added EGF, and in the presence of 10 μg/ml 225 mAb EGFR-blocking antibody. Errors represent ± SEM. Numbers above the bars are the number of individual cell tracks used in the analysis.

exogenous EGF. In contrast, WT and sEGF-expressing cells migrate at maximal speeds only when signaling by surface EGF–EGFR complexes is increased by addition of exogenous EGF.

Directional persistence of MEC migration is enhanced by autocrine EGF but reduced by exogenous EGF

In addition to effects on cell migration speed, EGF can strongly affect persistence of directionality (Ware et al., 1998). Such effects can be visualized qualitatively by reproducing sample cell movement paths on windrose plots (see Fig. 3). Fig. 3 A shows that EGF-Ct–expressing cells appear to travel in significantly more persistent paths in comparison to their paths in the presence of exogenous added EGF in Fig. 3 B. However, the mean speed of migration for the cells under these two conditions are not significantly different (Fig. 2).

To quantify the directional persistence property of cell migration, a parameter known as persistence time can be determined from the cell paths via the same sort of mathematical analysis by which the mean speed is obtained (Dunn and Brown, 1987). This parameter essentially represents the mean period of time over which a cell continues to move in a particular direction before changing direction by ~60 degrees. In accordance with the qualitative observations illustrated in Fig. 3, there is a striking quantitative effect of the various EGF presentation modes on HMEC directional persistence (Fig. 4). In the absence of exogenous EGF, EGF-Ct–expressing autocrine cells show a dramatically greater persistence time, ~50 min, com-

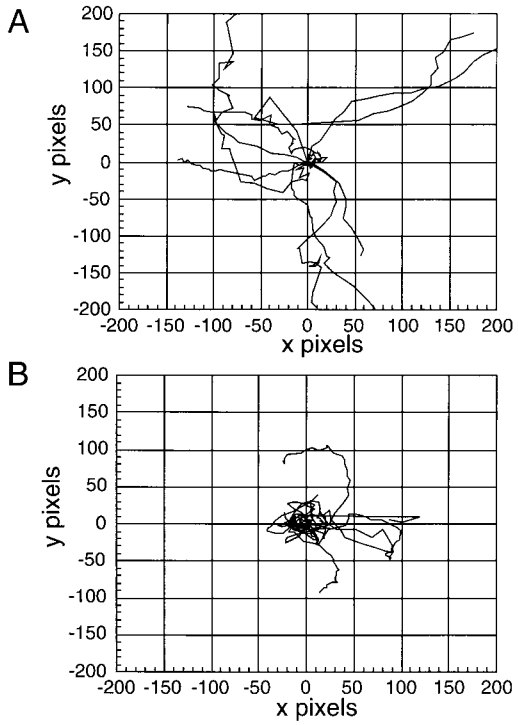

Figure 3. **Effect of ligand presentation on cell tracks of EGF-Ct expressing cells.** (A) Typical cell paths of cells expressing EGF-Ct in the absence of exogenously added EGF. Paths are of cells tracked over a period of 4–5 h and are replotted such that all paths start from the origin. (B) Cell paths of EGF-Ct–expressing cells in the presence of 2 nM exogenously added EGF. Cells shown in A and B have the same average cell speed, but have significantly different patterns of motion.

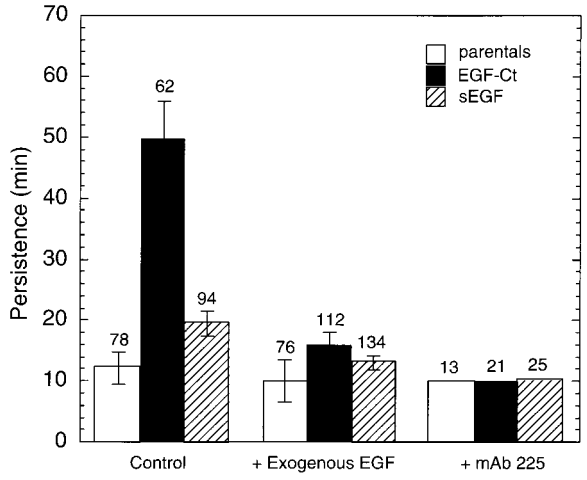

Figure 4. **Effect of ligand presentation on persistence time.** The cell tracks were analyzed using a persistence random walk model to obtain the value of persistence time. Filled, hatched, and blank bars represent the cell speeds of EGF-Ct–expressing cells, sEGF-expressing cells, and WT HMECs, respectively. Persistence times of the three cell types were calculated in the absence of exogenous EGF, in the presence of 2 nM exogenously added EGF, and in the presence of 10 μg/ml 225 mAb EGFR-blocking antibody. Errors represent ± SEM. Numbers above the bars are the number of individual cell tracks used in the analysis.

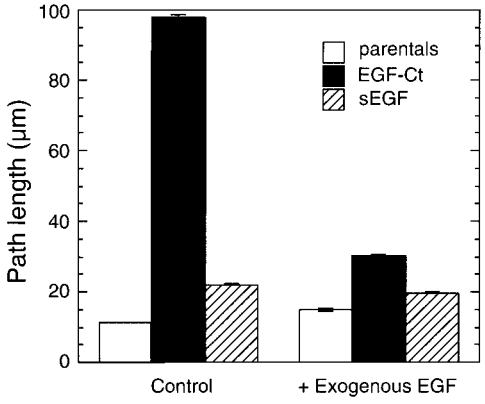

Figure 5. **Effect of ligand presentation on cell path length.** Path length is the product of cell speed and persistence under a given set of conditions. Filled, hatched, and blank bars represent the cell speeds of EGF-Ct–expressing cells, sEGF-expressing cells, and WT HMEC respectively. Path lengths were calculated in the presence and absence of exogenously added EGF.

pared with those for WT and sEGF-expressing HMECs of ~10 and 20 min, respectively. Addition of exogenous EGF abrogated the highly directional motion of the EGF-Ct cells, reducing their mean persistence time to ~15 min; persistence time of the WT and sEGF-expressing cells remained at this low level in the presence of the exogenous EGF. Blocking of cell-surface binding of EGF to EGFR using 225 mAb also resulted in reduced persistence time for the EGF-Ct cells with no effect on the WT and sEGF-expressing cells.

The net effect of EGFR-mediated induction of cell migration by the three modes of EGF presentation can be viewed in terms of a quantity reflecting the cell path behavior illustrated in the windrose plots of Fig. 3: the product of migration speed and persistence time is the "mean path length," plotted in Fig. 5. It is clear that EGF presented in autocrine manner provides HMEC with an ability to migrate for many cell lengths before altering their direction significantly, whereas presentation of EGF in intracrine or paracrine manner yields nondirected migration behavior which should result in cell scattering.

These findings indicate that directional persistence of cell migration is generated by EGF–EGFR signaling only under conditions of the ligand being released at the cell surface for subsequent recapture by plasma membrane receptors. The same quality of EGFR-mediated signals are apparently not generated by exogenous (or paracrine) ligand diffusing form the bulk medium to bind at the cell surface or by ligand binding intracellularly along the protein export pathway.

EGF presented in autocrine manner is restricted to the releasing cell

The above data suggests that spatial restriction in EGFR-mediated signaling is an important aspect of its role in cell migration. However, this implies that released EGF is restricted to the producing cell and does not affect all the cells in a population. To verify that the EGF-Ct autocrine loop indeed operates locally, we made visual observations of individual HMEC in mixed populations of EGF-Ct–expressing and WT cells. WT HMEC were labeled green and the EGF-Ct–expressing cells were labeled red using fluorescent vital dyes. Their paths were then tracked over a period of 4 h. As can be seen in the example photograph of Fig. 6, EGF-Ct–expressing cells exhibited strong locomotion behavior while WT cells, even those next to EGF-Ct-expressing cells, did not move significantly from their original locations. However, when exogenous EGF was added to the media, migration of WT HMEC was stimulated (in accord with data shown in Fig. 2), confirming that their motility apparatus was functional. The observation that autocrine EGF-stimulated migration of the EGF-Ct–expressing cells but not of neighboring WT cells supports the notion that the EGF autocrine loop is operating in spatially restricted, or "local," fashion. This is consistent with the predictions of a recent theoretical study that concluded that for typical values displayed by the EGFR autocrine system, a major portion of released autocrine ligand should be captured within a micron of the release point (Shvartsman et al., 2001).

Conclusions and speculation concerning operation of EGFR-mediated autocrine signaling loops

Together, the results of this study support the hypothesis that autocrine presentation of EGF stimulates a migratory cell behavioral response different from that stimulated by other modes of presentation of the same ligand. Only autocrine presentation yields a highly persistent cell locomotion response, whereas intracrine and exogenous/paracrine presentation produces a nondirectional locomotion response.

A possible mechanism underlying this directional regulation might be an asymmetry in the number of EGF–EGFR complexes across the cell, enhancing the probability that cell locomotion will continue in the direction of that asymmetry. This concept is consistent with earlier experimental and

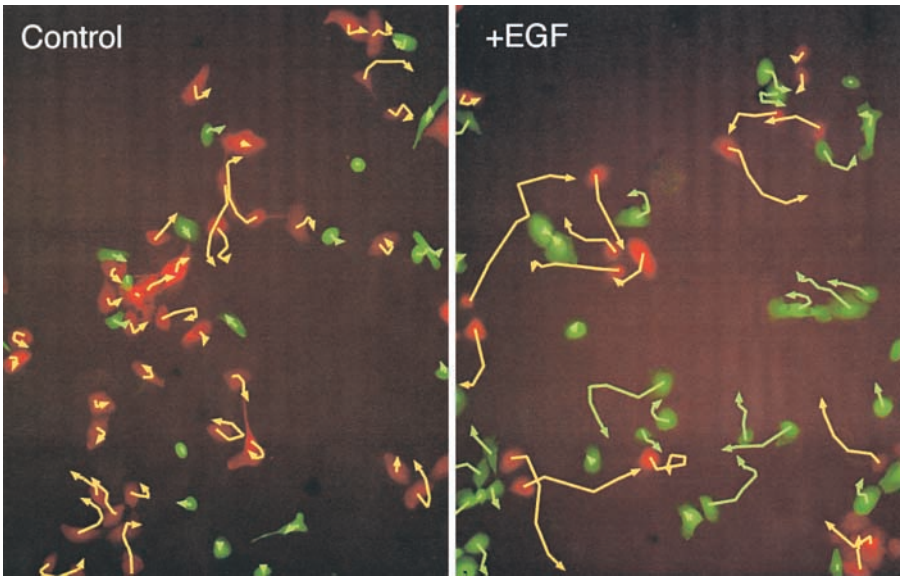

Figure 6. **EGF released from autocrine cells does not stimulate migration of neighboring cells.** WT HMECs were labeled with CellTracker green and cells expressing EGF-Ct were labeled with CellTracker orange. Cells were mixed overnight and followed for 4 h by two color fluorescence time-lapse microscopy using 4 min intervals. Shown are the initial images overlaid with trajectories marked at 40-min intervals. Left, cells in the absence of exogenous EGF. Right, cells in the presence of 2 nM exogenous EGF.

theoretical studies of neutrophil chemokinetic and chemotactic responses (Tranquillo et al., 1988), and may provide a means for more accurate sensing of environmental gradient cues, whether from soluble or matrix factors. One conceivable mechanism for this localized asymmetry could be a spatially restricted proteolytic cleavage of transmembrane EGF at particular locations on the plasma membrane stimulated by local activation of EGFR. Such a positive feedback mechanism is consistent with previous studies showing that EGFR ligands can stimulate their own proteolytic release (Baselga et al., 1996). In such a situation, addition of exogenous EGF would abolish the signaling asymmetry, resulting in the reduction of directional persistence as we have observed here. sEGF-expressing cells, on the other hand, would not have the ability to generate spatially restricted EGF–EGFR-signaling complexes because sEGF is constitutively active.

Our findings are also consistent with previous reports of divergence between EGFR-mediated motogenic and mitogenic signaling pathways at a cellular compartment level (Chen et al., 1994, 1996; Haugh et al., 1999), by which proliferation can be stimulated equally well by intracellular and cell-surface EGF–EGFR-signaling complexes, whereas a migratory response requires signaling at the cell surface. Previously, we had shown that 225 mAb does not block proliferation of cells expressing sEGF (Wiley et al., 1998). More recently, we have shown that most of activated EGFR-signaling complexes in HMEC are located in an intracellular compartment which would be poorly accessible to exogenous 225 mAb (Burke et al., 2001). Thus, our observation that 225 mAb is able to block the migration of sEGF expressers supports the idea that cell-surface EGF–EGFR complexes rather than intracellular complexes are principally involved in motility regulation of these cells. This is also consistent with of the observed differences in the ability of cell surface and internalized EGF–EGFR complexes to stimulate PIP_2 hydrolysis via PLC-γ1, which is known to be involved in EGF-stimulated motility (Chen et al., 1994; Haugh et al., 1999). Together, these results strongly suggest a role for compartmentalization of signaling molecules involved in cell migration and provide a mechanistic basis for the observed differences in the motility response of HMEC to autocrine versus intracrine EGF.

Finally, implications for potential therapeutic approaches to augmenting growth factor stimulation of cell function should be noted. If the physiological mode of growth factor presentation is autocrine, then provision of putatively therapeutic growth factor in exogenous or paracrine manner may well be counter-productive, as it would stimulate a cell response quite different from that generated by the normal autocrine presentation. In such a case, a gene-based delivery of the growth factor, targeted to the specific receptor-expressing cell type, would be the only feasible way to generate the desired physiological cell function.

Materials and methods

Cell lines and cell culture

HMEC type 184A1 were obtained from M. Stampfer (Stampfer and Yaswen, 1993; Stampfer et al., 1993). The construction and characterization of sEGF and EGF-Ct has been described previously (Wiley et al., 1998). Briefly, the sEGF and EGF-Ct constructs were packaged using the MFG retroviral vector and transfected into wild-type HMECs. For these studies, sEGF clone #1 and EGF-Ct clone #2 were used. These cells release 1.2 and 1.7 ng EGF per 10^6 cells/h, respectively, into the extracellular medium. The cells were cultured in DFCI-1 media as described previously (Band and Sager, 1989). Monoclonal antibody 225 mAb against the EGFR was purified from hybridoma supernatants as described previously (Wiley et al., 1998).

Migration assay

Cell migration speed was measured using time-lapse video microscopy of single cells. 30,000 cells were plated onto 35-mm dishes in 2.5 ml serum-free medium. 20 h postseeding, the medium was changed to 2.5 ml assay medium with or without 2 nM EGF and incubated at 37°C in humidified air for 2 h. At this cell density, soluble ligand concentration is relatively unchanged over a 24-h period (Reddy et al., 1994). 3 ml mineral oil was added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Ludl 99S008 motorized stage on a ZEISS Axiovert 35 microscope. Cell boundaries and centroids were identified using image processing software developed by Engineering Technology Center running under a LabVIEW (National Instruments) and Concept Vi environment. 5–10 cells/field in 10 different fields were scanned every 10 min for up to 6 h. The x and y coordinates of the cell centroids were recorded every 10 min.

Cell mixing experiments

Cells were stained before mixing with CellTracker fluorescent dyes (Molecular Probes, Inc.). Cells expressing EGF-Ct were stained with CellTracker green CMFDA and parental cells were stained with CellTracker orange CMTMR. The dyes were made as 10-mM stocks in DMSO and added to cells at a final concentration of 5 μM in serum-free growth medium for 30 min at 37°C. Cells were rinsed for 30 min, removed with trypsin and mixed at different ratios and densities, and cultured overnight in complete medium. Plates were visually selected to yield 1:1 orange:green cells at a low cell density. Fields of cells were recorded at 37°C on a heated stage for 4 h using both 488 and 562 nm excitation using a Nikon microscope automated with the Openlab software package (Improvision, Inc.). The migration speed and persistence of the cells was not altered by the staining protocol as compared with a parallel group of unstained cells.

Analysis of cell paths

Single cell speed was calculated by determining the total root mean squares path length, as measured by the total centroid displacement, divided by the tracking time (Dunn and Brown, 1987). The reported cell speed ± SEM for each condition is an average >200–250 cells. To calculate the persistence time, the cell paths were fit to a persistent random walk model. Average squared displacements were calculated for each cell using a method of nonoverlapping intervals. The plot of average squared displacements versus time for each cell was fit to the persistence random walk model using the value of speed of cell calculated independently, to obtain a value of persistence time of the particular cell. This value averaged >120–150 cells was reported as the persistence time ± SEM at the given conditions. Cell paths <4 h long were not included in the calculation of persistence time but were included in the calculation of cell speeds.

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