Review TRENDS in Cell Biology Vol.13 No.1 January 2003

Computational modeling of the EGF-receptor system: a paradigm for systems biology

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Computational models have rarely been used as tools by biologists but, when models provide experimentally testable predictions, they can be extremely useful. The epidermal growth factor receptor (EGFR) is probably the best-understood receptor system, and computational models have played a significant part in its elucidation. For many years, models have been used to analyze EGFR dynamics and to interpret mutational studies, and are now being used to understand processes including signal transduction, autocrine loops and developmental patterning. The success of EGFR modeling can be a guide to combining models and experiments productively to understand complex biological processes as integrated systems.

Epidermal growth factor (EGF) has just turned 40. From its original description as a factor that induced precocious eyelid opening in newborn mice, EGF has been used to explore and define what is arguably the best-understood of all receptor systems. The epidermal growth factor receptor (EGFR) system was the first found to have tyrosine-kinase activity and has been used in pioneering studies of fundamental processes such as receptor-mediated endocytosis, oncogenesis, mitogen-activated-protein-kinase (MAPK) signaling pathways, multi-receptor-family interactions, autocrine loops and receptor transactivation [1]. The EGFR and other members of its family have been the targets of successful therapies against cancer and continue to be sources of discoveries about the cell signaling mechanisms involved in development, tissue homeostasis and disease [2,3].

Although the EGFR system's role in understanding signal transduction is well appreciated, it has also played a role in shaping the nascent field of systems biology. The recent flood of genomic and proteomic data has spurred efforts to integrate this information into a higher-order, systems-level understanding of how organisms function [4]. Clearly, there is an urgent need to combine computational models with molecular-level data to improve our understanding of complex cellular functions. It is not always obvious, however, how to build models that are experimentally testable and that can accurately recapitulate cellular functions. The EGFR system has been an extremely useful test case for modeling. It is present at substantial levels in a range of different cell types, and high-quality antibodies and molecular reagents have been widely available for the past 20 years. This has enabled a range of quantitative studies to be performed. In parallel with the use of these molecule-level tools, models of different aspects of the EGFR system have been built. In many respects, the EGFR system has served as a test case for the utility of a systems approach to understanding biological processes. In this article, we discuss the useful role that modeling has played in understanding the EGFR system and how this experience can guide the building of useful cellular models in the future.

Binding and trafficking

The original models of EGFR binding and internalization were derived from classic enzyme kinetics [5–7]. Receptor-mediated endocytosis was modeled as an enzymatic process in which surface-associated EGF was considered to be a 'substrate', internalized EGF was the 'product' and coated pits were the 'enzyme'. This modeling approach was experimentally accessible because of the availability of radiolabeled EGF and the discovery that a simple acidic cell wash could selectively release surface-associated ligands without affecting internalized ligands. Thus, the modeled substrates and products could readily be followed over time, permitting validation of enzymology-based models [7].

These steady-state and kinetic models were very successful at capturing many of the dynamic features of EGF binding, internalization and degradation (Fig. 1) and they were used as a basis to follow the pharmacokinetics of EGF distribution in the whole animal [8]. They have also been used to investigate a wide range of growth factor, hormone and cytokine systems [9]. They could accurately predict complex binding and distribution patterns of ligands within cells and helped to establish relationships

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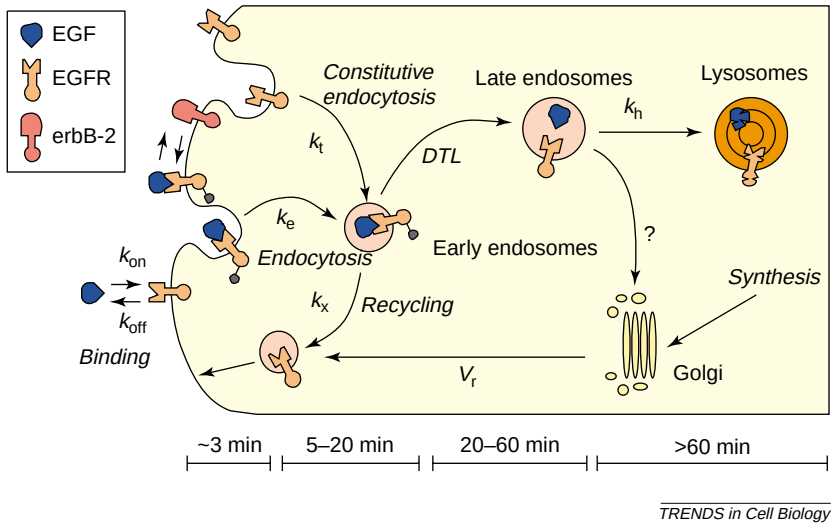

Fig. 1. Trafficking dynamics of the epidermal growth factor (EGF) receptor (EGFR) system. EGF or a homologous ligand binds reversibly to the receptor (rate constant of association is k_{on} and that of dissociation is k_{off}), which is then internalized. The rate constant of endocytosis is k_e and that of recycling is k_x. Empty receptors are constitutively internalized with a rate constant of k_t. New receptors are continuously brought to the cell surface at a rate of V_r. Receptors can interact reversibly with other members of the EGFR-family, such as ErbB-2. Occupied receptors can also be transferred to late endosomes and lysosomes for degradation with a rate constant of k_h. There is a degradation lag time (DLT) between initial internalization and ligand degradation that can be modeled as a queue. There is a continual flux of receptors and ligand through different compartments, so multiple regulatory steps dictate their overall dynamic distribution. Occupied receptors only spend a relatively short time at the cell surface owing to rapid internalization. Thus, intracellular compartments can contain substantial levels of occupied, kinase-active receptors.

between physiological responses such as mitogenesis and levels of receptor activation in specific cellular compartments [10–13].

These early studies demonstrated that the EGFR system behaves as a robust, proportional control system. Thus, even though many complex processes occur in response to EGFR activation (e.g. induced internalization, desensitization and affinity shifts), the relationship between the system input (ligand availability) and the final output (cell responses) is remarkably simple. Simple behavior that arises from complex processes is characteristic of robust systems [14]. From this perspective, the mechanistic detail of receptor behavior should be interpreted in the context of the overall system behavior.

Modeling the EGFR system as a pseudo-enzymatic reaction took advantage of 50 years of research in enzyme kinetics. For example, cells that overproduce the EGFR could be modeled as a system in which the substrate was in great excess of the enzymes, thus allowing detection of additional rate-limiting steps [15,16]. This feature was invaluable for dissecting the mechanisms of occupancy-induced endocytosis and downregulation. For example, studies of mutated EGFR demonstrated that eliminating its intrinsic receptor-tyrosine-kinase activity profoundly altered receptor distribution between the surface and interior of cells but had no significant effect on the net rate of EGF uptake by cells [17]. Two research groups interpreted these observations in different ways. One group suggested that an alteration in receptor recycling was responsible, whereas the second group insisted that a change in receptor internalization was the mechanism [18,19]. Mathematical modeling clearly favored the internalization-based hypothesis. The ensuing decade of research verified the internalization-based mechanism predicted by the EGFR trafficking models. This success prompted the use of modeling in mapping functional EGFR domains. In fact, the EGFR domains responsible for both binding to coated pits and lysosomal targeting were mapped largely by using mathematical models [20–22].

Models have allowed us to appreciate the complexity of EGFR trafficking and have provided significant insight into its regulation. However, these models have been useful only because of the parallel development of powerful molecular, microscopic-imaging-based and biochemical kinetic assays that enable their testing and refinement. Site-directed mutagenesis of the EGFR and its ligands is a particularly powerful way to test these models because it allows a selected part of the overall trafficking pathway to be modified. Labeled antibodies to the receptor have also been useful as specific tags to follow the behavior of empty receptors to complement investigations of activated ligand-bound receptors [22,23]. These studies have shown that there are multiple trafficking 'decision points' along the endocytic pathway (Fig. 1) that are controlled by the state of receptor occupancy, receptor kinase activity or other regulatory processes such as ubiquitination [24,25]. Different ligands that activate the EGFR differ in their ability to bind to the receptor within the acidic lumen of endosomes and thus have distinct effects on intracellular receptor routing [26].

EGFR trafficking is not a single pathway from the surface to the lysosomes but rather a recursive process with significant recycling of both the ligands and the receptor through multiple cellular compartments [24]. The rates of individual processes and the levels of

specific adaptor proteins also depend on the specific cell type [23]. Thus, the dynamic distribution of cellular EGFR reflects ligand type, receptor levels, constitutive endocytosis and activity of other regulatory molecules [24,27].

The past five years have witnessed an explosion of descriptive studies asserting that one molecule or another 'mediates downregulation' of the EGFR. These studies generally examine total receptor levels at the cell surface at only a few time points and so it is difficult to know at which trafficking step they operate. Because of the complexity of EGFR trafficking, we will not fully understand the regulatory roles of these molecules until experiments are coupled to computational models. Recent studies of individual trafficking steps using powerful new molecular tools have verified the predictions made by the mathematical models more than a decade earlier, showing the validity of this approach [28].

Signaling and functional responses

Considerable insights have been gained from combining quantitative experimental and modeling approaches to EGFR trafficking problems. An important benefit of quantitatively understanding EGFR trafficking dynamics is the improved ability to interpret data on cells' functional responses and the signal transduction processes governing them. As one simple example, the relationship between external concentrations of EGF and cell proliferation appears to be complex (as characterized by measures such as DNA synthesis rate or increase in cell number). However, mathematical modeling of the cell-level binding and trafficking dynamics has demonstrated that the underlying basis of this dependence is actually a linear dependence between cellular responses and the number of EGFR–ligand complexes at steady-state [11], at least for fibroblasts and epithelial cells that express receptors in the physiological range [29]. Therefore, the typical sigmoid relationship between ligand concentration and proliferation arises only indirectly from the linear dependence on receptor–ligand complex number convoluted by binding dynamics and simultaneous receptor downregulation and ligand depletion.

This insight ultimately led to a reinterpretation of the finding [30] that an EGFR mutant with normal kinase activity but reduced internalization rates causes increased cell proliferation. Instead of the original explanation (enhanced receptor signaling at the cell surface), it was determined that a decrease in receptor downregulation and ligand depletion could account entirely for the overall proliferation response of the mutant receptor [31]. The linear dependence of proliferation on ligand–receptor complex number remained the same for cells expressing mutant or wild-type receptors, but the number of complexes remained at higher levels longer for the mutant receptor.

Using models to 'deconvolute' relationships between experimentally accessible parameters, such as initial ligand concentrations and important cellular parameters (e.g. the number of occupied receptors) is one of their most useful, if often overlooked, features. For example, it was known for years that the addition of heparin, a glycosaminoglycan, could greatly enhance the biological activity of fibroblast growth factor, but the mechanism of this enhanced activity was obscured by the many parameters affected by heparin (e.g. receptor affinity and ligand stability). By using general models derived from the EGFR system and experiments to measure important system parameters, it was demonstrated that a simple enhancement of the number of occupied receptors by heparin was responsible [32]. Thus, models can show that seemingly complex effects can have simple explanations.

Models can also be used to make non-intuitive predictions. For example, cell responses to EGF depend on the number of occupied receptors as well as their persistence. Natural ligands appear to have very short half-lifes in cells, probably to allow for rapid regulation of cell behavior. If an EGFR ligand had a low affinity, it would occupy fewer receptors but would persist longer because of its inability to drive occupied receptors to lysosomes. Theoretically, we could re-engineer a ligand with a lower affinity that would consequently have a significantly higher biological activity because of the increased receptor and ligand recycling [12]. These predictions were borne out by subsequent experimental studies with the Y13G EGF mutant [33]. This exciting, mathematical model-derived prediction of how to 're-engineer' a growth factor or cytokine to generate a superior functional response has been extended more broadly to other ligand, receptor and cell systems, offering a general insight into receptor–ligand dynamic operation in cell physiology [34,35]. Functionally, we might conclude that natural ligands have evolved for optimal regulation rather than for maximal activity.

Quantitative analysis is useful for understanding how the myriad intracellular signaling pathways activated by EGFR can regulate cell functional responses and not only proliferation but also survival, migration and differentiation. Because there is a linear dependence of proliferation on the number of steady-state EGFR–ligand complexes, we can easily calculate the magnitude of mitogenic signaling on a per-receptor basis. Diverse cell types with disparate signaling networks (which could be established by gene-expression studies) should show different amounts of signaling arising from an activated receptor complex [12]. To explore this connection rigorously, we must begin to develop analogous, dynamic mathematical models – accompanied by appropriate quantitative experimental studies – for the key signaling pathways downstream of EGFR. Such efforts have been made by several laboratories [36–39].

It is essential, of course, that signaling models be coupled with binding and trafficking models to capture correctly the effects of receptor distribution on signaling pathway activities. The model of Schoeberl *et al.* is the most comprehensive in that it includes the fullest range of dynamic processes and makes several interesting and significant predictions that can be validated by experimental tests [39]. Chief among these is the prediction that the activation amplitude of the MAPK

extracellular-regulated kinase (ERK) is a non-linear function of EGFR–ligand complex levels. One implication is that ERK-mediated cell responses might be surprisingly difficult to suppress by blocking EGFR ligand binding (e.g. using anti-EGFR antibodies) or inhibiting EGFR kinase activity (e.g. with small-molecule kinase inhibitors). In any event, pursuing this kind of combined modeling and quantitative experimental approach with inclusion of binding, trafficking and signaling processes could significantly enhance efforts to identify useful drug targets.

Interactions between EGFR-family members

The EGFR can form homodimers with itself and heterodimers with other members of its receptor-family, such as ErbB-2 [40]. Forming multimers can significantly affect receptor behavior such as ligand affinity and these processes have been extensively modeled. Interestingly, modeling of receptor dimerization demonstrated almost a decade ago that the multiple affinity forms of this receptor could not be explained by occupancy-induced dimerization [41]. Instead, the models suggested that either dimers were preformed or EGF binds only to monomeric receptors that subsequently form dimers. Support for these predictions have recently been obtained by quantitative fluorescence microscopy [42] and from the crystal structure of the ligand–receptor complex [43].

Overproduction of ErbB-2 can have pronounced effects on the activation and downregulation of the EGFR, presumably resulting from a shift of the normal distribution of EGFR homo and heterodimers to favor the EGFR–ErbB-2 heterodimers. These effects can be surmised to arise solely from differences in signaling pathway partners among the various heterodimers, but the situation is not nearly so simple as this because of the concurrent alterations of binding and trafficking behavior of the receptor-family members involved. The mechanisms by which ErbB-2 overproduction alters the normal dynamic behavior of the EGFR have been analyzed by both modeling and experimental approaches [44,45], and these analyses suggest that several different binding and trafficking steps are affected, resulting in a net increase in EGFR activity. Such enhanced activity could contribute to the transforming activity associated with ErbB-2 overproduction. Because many different aspects of the EGFR pathway are affected, including binding, internalization and recycling, the overall effect of ErbB-2 overproduction should depend on factors such as the EGFR production level, the available ligand types and the cellular endocytic activity. This kind of quantitative analysis will have to be performed in rigorous depth to interpret the mechanisms by which ErbB-2 production affects overall cellular responses. Although models have primarily been used to analyze the effect of ErbB-2 overproduction, they could also be used to predict the biological effect of any level of production and provide insight into the design of more-effective therapies designed to disrupt ErbB signaling.

Autocrine signaling

The EGFR itself is only a single part of the overall EGFR system. Within an organism, receptors are part of a system that includes the cognate ligand and all of the regulatory components controlling upstream aspects of ligand availability and downstream signal transduction. Although models have been useful for understanding EGFR dynamics, they are also proving to be essential for understanding the overall operation of the EGFR system. Autocrine signaling is an excellent example of how a mathematical model can be applied to a system that appears to be almost overwhelmingly complex at first glance and show that it operates by simple principles.

We know that most cells that depend on the EGFR system for regulated proliferation synthesize both EGFR and a cognate ligand, and can thus be classified as autocrine systems (Fig. 2a–c). Although it was originally thought to be associated with cell transformation, autocrine signaling is now known to be a fairly common cell regulatory system. Unfortunately, the recursive, 'closed loop' nature of these systems makes them very difficult to investigate by standard experimental approaches [46]. The existing models of EGFR dynamics, however, provide a solid foundation on which to build more-complex autocrine models.

Several different approaches have been used to model autocrine systems. Stochastic and deterministic techniques have been used to characterize the diffusion and capture of autocrine ligands with the intent of creating models in which crucial parameters could be measured experimentally and thus validated. Brownian-motion theory and computer simulations were used to calculate the trajectories of secreted ligands, whereas continuum reaction–diffusion equations were used to characterize the fluxes of autocrine ligands. Both approaches suggested that autocrine loops formed by EGFR and its ligands could be highly localized and even restricted to specific areas of a single cell's surface [47,48]. More important, parameters that controlled the distance of autocrine loops, such as receptor density, ligand diffusion and receptor affinity, are actively regulated by cells or are defining characteristics of different EGFR ligands (Fig. 2d,e). This suggests that cells can actively control the distance over which they broadcast autocrine ligands and that travel distance is an important regulatory characteristic.

These autocrine models have been validated by experimental studies, primarily by the use of reconstructed autocrine cells in which ligands and receptors of defined structure have been introduced into normally EGFR-negative cell lines [29,46,49]. By using regulated gene-expression systems and instruments for continuously monitoring cell responses, the properties of autocrine EGFR signaling have been explored extensively. These studies have revealed some remarkably simple systems behavior. For example, autocrine ligands that bind with high affinity, such as transforming growth factor-α, rarely escape from the cells that produce them but are instead consumed locally. In agreement with models, these studies showed that the extent of EGFR occupancy is proportional to the ratio of ligand-production and receptor-production rates [29]. Thus, cells respond to autocrine ligands as a linear function of their production (Fig. 3a). In the case of

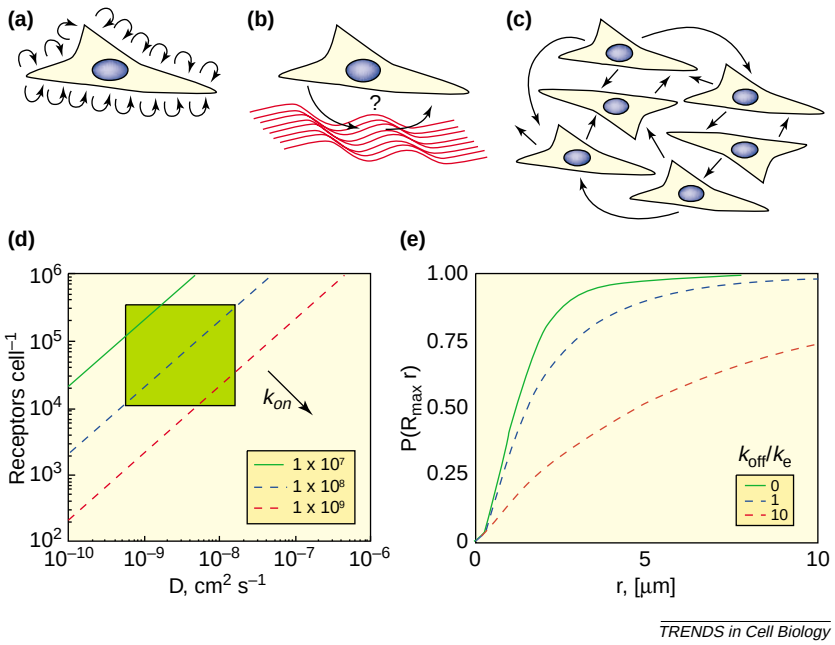

Fig. 2. Autocrine signaling can act at different spatial ranges. Three proposed modes of autocrine signaling are considered here. (a) Cell sonar, in which the spatial scale is much less than the cell diameter. (b) Microenvironment sensing, in which the factor can be lost into the extracellular matrix before rebinding. (c) Community effect, in which a group of cells actively exchange factors that influence their behavior. (d) The spatial scale of autocrine loops depends on receptor number (*y* axis), ligand diffusivity (*x* axis) and the rate constant of association. The calculated conditions for >95% ligand capture by the producing cell are shown at three different association rate constants (k_{on}) (shown in units of M^{-1} min^{-1}). For parameters left of a given line, the ligand is captured. To the right of the line, ligand escapes. The highlighted box is the range of diffusivity and receptor number usually observed for epidermal-growth factor-receptor (EGFR) ligands and EGFR-bearing cells. Observed EGFR association rate constants usually fall between 2×10^7 M^{-1} min^{-1} and 5×10^8 M^{-1} min^{-1}, indicating a cell-sonar-signaling mode. Variations in parameters such as receptor–ligand affinity and receptor numbers could switch cells between different autocrine modes. (e) The effect of endocytosis on the spatial range of signaling. The distance traveled by a ligand (*x*-axis) and the probability of capture at that travel distance (*y*-axis) are shown at three different ratios between the ligand-dissociation rate constant and the endocytosis rate constant (k_{off}/k_e). Most commonly, this ratio is ~1. Thus, rapid receptor endocytosis restricts the spatial scale of autocrine loops.

ligands that must be proteolytically released for activity, this means that cell response is proportional to the rate of ligand release. Other studies have shown that the distance traveled by a ligand is indeed controlled by its affinity for the EGFR [50]. Thus, high-affinity ligands such as transforming growth factor-α appear to be short-range ligands, whereas low-affinity ligands such as amphiregulin probably act at longer distances.

The linearity and distances of autocrine responses are fairly simple predictions of autocrine models, but they can also be used as the basis of more non-intuitive predictions. For example, we know that EGF stimulates cell motility and that EGFR activation stimulates the proteolytic release of autocrine ligands [2,51]. The physical parameters of the EGFR autocrine system in human mammary epithelial cells suggest that autocrine loops should be localized to a small section of the cell surface [48]. Thus, we could predict that regulated autocrine signaling in these cells should enhance directional migration but that addition of exogenous EGF or expression of a constitutively active autocrine loop should decrease directional migration. These predictions were recently experimentally validated, suggesting a mechanistic basis for how EGFR might help to regulate important developmental processes including branching morphogenesis and cell migration [52].

A single autocrine cell can modulate signals over time, but a layer of autocrine cells can process signals in both time and space. In this way simple signals can be converted into complex spatiotemporal patterns. Pattern formation mediated by autocrine EGFR signaling has been well studied in *Drosophila* development [53]. Recently, we have started to build mechanistic models of autocrine-mediated pattern formation during *Drosophila* oogenesis [54]. Our models have demonstrated that modulating the physical parameters associated with distributed autocrine loops can yield a variety of the observed tissue patterns, supporting specific mechanisms proposed on the basis of genetics and biochemistry.

Transactivation and positive feedback

One of the most interesting aspects of autocrine EGFR signaling is that ligand processing and release are regulated by extracellular signals. In particular, Ras–MAPK signaling and intracellular calcium levels are major regulators of EGFR ligand release [55,56]. This appears to be the main mechanism responsible for EGFR 'transactivation' by other hormones (Fig. 3b). However, these signaling pathways are also activated by the EGFR itself. Combined with the high probability of autocrine-ligand recapture, this means that the pathways responsible for ligand release, extracellular transport and signaling can form a positive feedback loop. Such a loop from EGFR ligand binding to ligand release has been

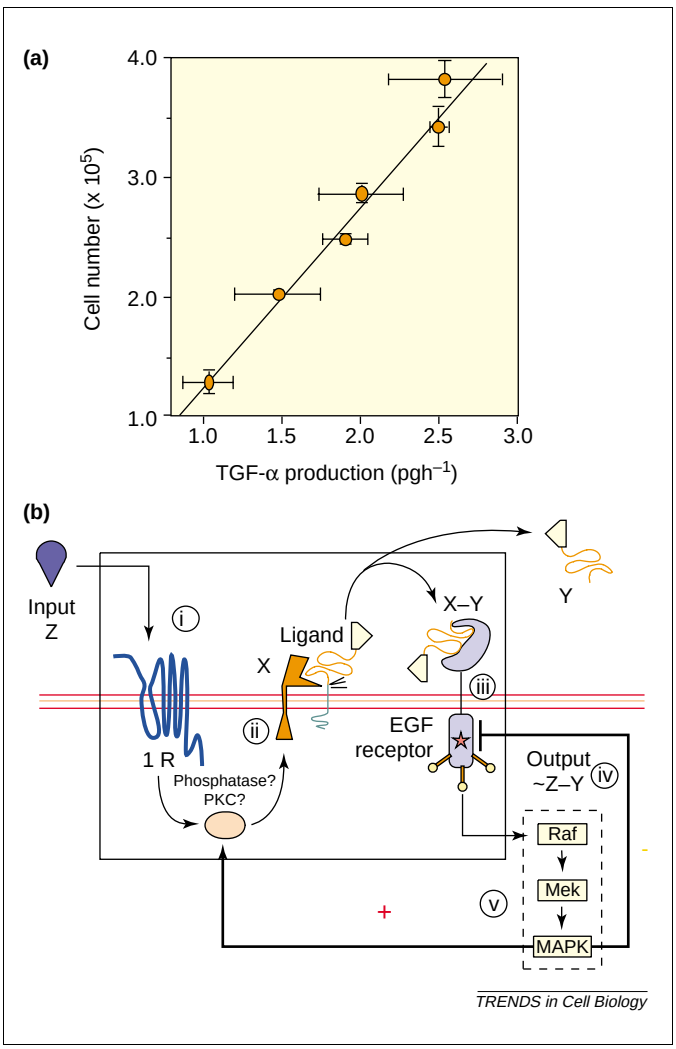

Fig. 3. The epidermal growth factor (EGF) receptor as a linear control system. (a) Production of transforming growth factor-α (TGF-α) was modulated in autocrine mammary epithelial cells by use of the metalloprotease inhibitor Batimastat. EGF-receptor-dependent proliferation of the cell was then determined. Proliferation is linearly dependent on ligand-production, consistent with the properties of a proportional control system. (b) Model for 'microenvironment-coupled' autocrine signaling. The input is a transactivating ligand (such as angiotensin) that appears at the rate *Z* and stimulates the primary receptor (R), leading to specific increase in intracellular signal mediators (i). This increases the rate of EGF release (ii). If the new rate of ligand release is *X* and the rate of ligand loss from the system is *Y* then the rate of receptor occupancy will be $X - Y$ (iii). The lost ligand could also coordinate the response of other cells in the population. The final output is proportional to the initial input *Z* minus the amount lost *Y* (iv). Downstream components of the signaling cascade can then regulate receptor activity and ligand release by both negative and positive feedback (v).

demonstrated in several developmental and pathological settings [53,57].

Positive autocrine feedback loops can profoundly affect cell physiology. Radiation responses of several autocrine carcinoma cell lines provide a striking example [57]. It was found that instead of inducing cell death, a pulse of ionizing radiation could stimulate cell proliferation by inducing autocrine EGFR signaling, leading to long-lasting and even sustained ERK2 activity [58,59]. Inhibiting this long-lasting MAPK response significantly enhanced the toxicity of radiation to several carcinoma cell lines. These observations provide a rationale for several improved anti-cancer protocols in which radiation treatment is combined with molecular manipulations that antagonize autocrine EGFR signaling [60]. Recently, we have described an autocrine-signaling model with positive feedback that successfully predicts the long-lasting secondary excitations in the MAPK radiation responses. These probably arise from strongly non-linear interactions between ligand release, extracellular transport, binding and intracellular signaling [61]. The model also accounts for the qualitative differences in MAPK responses (sustained versus transient activation) observed in cell lines with different numbers of EGFRs [57–59]. Given the many molecular components composing autocrine loops, therapeutic protocol designs would probably benefit from this type of mechanistic modeling.

Future directions: integrated models

We have described here many of the models of the EGFR system that have been built and experimentally validated during the past 20 years. Some of these models have been used to analyze the system and to predict new and unexpected properties. So, what have we learned and how should this guide our future efforts in systems biology? First, we have learned that models are only useful if experiments can be designed that directly test specific predictions of the model; in other words, the predictions must be in a form that can be directly measured in an experiment. A model that predicts an increase in receptor dimerization, for example, is only useful if we can actually measure that parameter. The slow progress in building comprehensive models of the EGFR system is primarily due to the difficulty of experimental design and execution. A model that takes only a few weeks to construct might take years or even decades to test adequately.

Second, we have found that as models become more complex, the amount of data necessary to validate them becomes greater than can be generated by usual laboratory experiments. This experimental constraint has tended to keep the models small. So, we have models of trafficking, signaling, heterodimerization and so forth, but it will be very difficult to combine these into larger, integrated models until we can determine the best experimental way to validate such complex constructs. This is where the new, high-throughput data-generation approach espoused by systems biology promises to have the greatest impact. Computer-based models can be used as 'high-throughput hypotheses' to exploit these high-throughput data-generation techniques, such as advanced imaging or mass-spectrometry-based proteomics [4].

Finally, the ultimate test of any model is whether it can provide a useful higher-level perspective of a complex problem. From this viewpoint, we believe the EGFR models have been a resounding success. For example, we have learned that receptor trafficking controls the information flux through cells. Cell-surface signaling represents the instantaneous information presented to the cell, whereas endosomal signaling represents the integration of information over many cycles of cell-surface binding [62–64]. We have found

that ligand availability is the master regulator of the EGFR system and that regulated ligand proteolysis controls virtually all downstream receptor activities [65,66].

Concluding remarks

The EGFR system appears to behave as a proportional control system in which signal processing occurs at many different points in the pathway. It is clear that cells 'filter' the information that they receive before passing it to the nucleus. The many different control points in the EGFR pathway make it an excellent system for investigating how cells process contextual information. We expect that computational models will be essential for understanding this process. Fortunately, the days of 'early retirement' are over and the next 40 years of research on the EGFR system might yet prove to be as exciting as the first 40!

Acknowledgements

Our work has been supported over the years by the National Institutes of Health, the National Science Foundation, the US Department of Defense and the US Department of Energy.

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