

An integrated agent-mathematical model of the effect of intercellular signalling via the epidermal growth factor receptor on cell proliferation

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Abstract

We have previously developed *Epitheliome*, a software agent representation of the growth and repair characteristics of epithelial cell populations, where cell behaviour is governed by a number of simple rules. In this paper, we describe how this model has been extended to incorporate an example of a molecular ‘mechanism’ behind a rule—in this case, how signalling by both endogenous and exogenous ligands of the epidermal growth factor receptor (EGFR) can impact on the proliferation of cell agents. We have developed a mathematical model representing release of endogenous ligand by cells, three-dimensional diffusion of the secreted molecules through a volume of cell culture medium, ligand–receptor binding, and bound receptor internalization and trafficking. Information relating to quantities of molecular species associated with each cell agent is frequently exchanged between the agent and signalling models, and the ratio of bound to free receptors determines cell cycle progression and hence the proliferative behaviour of the cell agents. We have applied this integrated model to examine the effect of plating density on tissue growth via autocrine/paracrine signalling. This predicts that cell growth is dependent on the concentration of exogenous ligand, but where this is limited, then growth becomes dependent on cell density and the availability of endogenous ligand. We have further modified the calcium concentration of the medium to modulate the formation of intercellular bonds between cells and shown that the increased propensity for cells to form colonies in physiological calcium does not result in significantly different patterns of receptor occupancy. In conclusion, our approach demonstrates that by combining agent-based and mathematical modelling paradigms, it is possible to probe the complex feedback relationship between the behaviour of individual cells and their interaction with one another and their environment.

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1. Introduction

Signalling via the epidermal growth factor receptor (EGFR) is known to be an important mediator of growth in many tissue types. In particular, autocrine signalling, whereby cells produce and respond to the same ligand, has been demonstrated to be critical in controlling the growth and repair characteristics of several epithelial cell types, including keratinocyte (Stoll et al., 1997; Kansra et al., 2004), urothelial (Freeman et al., 1997; Varley et al., 2005), bronchial (Chu et al., 2005), corneal (Xu et al., 2004) and

mammary (Maheshwari et al., 2001) cells. Dysregulation of EGFR-mediated signalling has also been implicated in malignancy (Salomon et al., 1995; Normanno et al., 2006). Hence understanding the key components and interactions of the autocrine signalling loop is an important step towards promoting normal tissue regeneration and inhibiting malignant growth.

EGFR signalling can be regulated at a number of different levels: the production of multiple soluble ligands by metalloproteinase cleavage, ligand-binding and activation of receptors, and downstream signalling cascades leading ultimately to a cellular response. The system is regulated at each stage by feedback loops, leading to changes in receptor and ligand availability. In addition, the

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spatial range of the signal will be governed by the ability of a cell to recapture the ligand released. Ligands that escape from the parent cell will diffuse through the medium where they may become bound to the extracellular matrix, or activate receptors on the surface of neighbouring cells of the same type—a process referred to as ‘homotypic paracrine’ signalling. Hence autocrine/homotypic paracrine signalling can be considered a mechanism by which cells exchange information with one another, and also probe their environment. It has been demonstrated that in many cases, the autocrine ligand may be active in the membrane-bound form, and can activate receptors on adjacent cells—a process known as juxtacrine signalling. Juxtacrine interactions are not explored in this paper, but are a natural future extension to our model.

Activation of EGFR results in downstream activation of the ERK mitogen-activated protein kinase (MAPK) cascade that regulates proliferation through the transcriptional regulation of cyclins, which mediate S phase entry (Wells, 1999), and the PLC γ pathway, which controls cell motility. Crosstalk exists between these and other pathways, which in effect means that there is a complex relationship between receptor activation and cellular response, modulated by a range of cellular and environmental factors. In particular, interactions between the EGFR-mediated MAPK and integrin signalling pathways are critical in modulating cell cycle progression, arrest and apoptosis (Roovers and Assoian, 2000). The details of the events initiated by EGFR binding and phenotypic change remain unclear; however, Knauer et al. (1984) used a combination of experiment and computational modelling to relate receptor occupancy to mitogenic response in human fibroblasts and demonstrated that the maximum mitogenic response occurred when 25% of the initial surface receptors were occupied.

Mathematical modelling is a useful tool that can be applied to probe the non-intuitive behaviour of complex biological systems. A number of models have been developed to study different aspects of EGFR signalling, mostly at the level of a single cell. Several of these models incorporate receptor–ligand binding, internalization and signalling, but do not include ligand release or diffusion, and thus cannot be applied to systems where autocrine signalling is important (Kholodenko et al., 1999; Schoeberl et al., 2002; Resat et al., 2003). Stochastic models based on Brownian dynamics have been developed to study autocrine and paracrine signalling processes in cell cultures and tissues, though trafficking of internalized ligands has not been explicitly included (Batsilas et al., 2003; Berezhkovskii et al., 2004). Other workers have focussed on ligand release, binding and trafficking, but have not considered the activation of intracellular signalling pathways (Oehrtman et al., 1998; DeWitt et al., 2001; Pribyl et al., 2003; Monine et al., 2005). The most comprehensive models address autocrine receptor–ligand dynamics as well as intracellular signalling via the MAPK pathway (Shvartsman et al., 2001; Maly et al., 2004). The detail of these

purely mathematical models renders them highly powerful in their ability to probe the signalling network at many levels; however, the key element missing is the effect of the signalling on cell behaviour (i.e. the link between molecular dynamics and cellular response or phenotype). Thus, it is not possible to probe the link between signalling mechanisms and the characteristics of a cell population. A model incorporating the results of Knauer and colleagues was developed by Starbuck and Lauffenburger (1992), which related mitogenic response to receptor occupancy and allowed the prediction of a growth curve for a population of fibroblasts. More recently, Athale et al. (2005) incorporated a grid-based agent model with an ordinary differential equation (ODE) model of autocrine TGF α binding and downstream PLC γ signalling. This was used to examine the switch between migratory and proliferative phenotypes in glioma cells, although diffusion of the ligand between cells was not explicitly modelled.

Our ultimate aim is to develop a computational paradigm that can simulate biological processes from the molecular, through to the cellular, and ultimately the tissue level. We have chosen to use a ‘middle out’ approach to modelling in that our framework has initially been developed at the level of the individual cell, and can be extended to incorporate molecular to tissue-scale phenomena. In this paper, we describe how we have extended our existing phenomenological rule-based agent model (Walker et al., 2004a, b) in order to include the mechanistic aspects of autocrine and paracrine growth factor signalling via the EGFR receptor. This is achieved by integrating a 3D numerical reaction diffusion trafficking model, which regularly exchanges information with the agent environment. The results of this numerical model feedback into the agent environment by influencing the decision of agents whether or not to traverse a cell cycle checkpoint. As an exemplar study, we have modelled population growth in monolayer cell culture of normal epithelial cells and examined the relationships between amount of available exogenous growth factor, the volume of the culture medium and cell density. Our in vitro model incorporates rules for modulating the exogenous calcium concentration of the medium with consequential effects on intercellular junction formation and we have used this to predict how this affects the growth factor availability and hence expansion of cell populations with qualitatively different growth patterns. The approach demonstrates that by combining different modelling paradigms it is possible to probe the complex feedback relationship between the behaviour of individual cells and their interaction with one another and their environment.

2. Methods

2.1. The agent modelling paradigm

We have previously developed *Epitheliome*, an agent-based model of epithelial cells, which we have applied to

simulating growth (Walker et al., 2004b) and repair (Walker et al., 2004a) in monolayer urothelial cell cultures, and more recently, stratification and differentiation in normal and transformed keratinocytes (Walker et al., in press). Software agents represent individual cells, and iteratively change their state (e.g. cell cycle position) according to a number of pre-programmed rules representing biological behaviour such as proliferation, intercellular adhesion, migration and apoptosis. Agents are not confined to a grid and the mechanics of the system is explicitly solved in order to prevent agent overlap and achieve realistic patterns of growth. This model is described in detail elsewhere, and the key rules, representing various aspects of cell behaviour in monolayer cell culture, are shown schematically in Fig. 1.

The *Epitheliome* model can be considered as a phenomenological representation of actual cell behaviour, as the rules governing state transitions are based purely on observations made experimentally, many of which are published in the literature. Previously, we have not

attempted to explicitly relate these rules to the underlying mechanisms. For instance, agents progress around the cell cycle consisting of phases representing G1, S, G2 and M, and traverse a single checkpoint midway through G1. To date, this checkpoint was dependent on cell bonding and cell shape: cells with more than n bonds with adjacent neighbours (assuming one virtual bond per cell pair, representing multiple adherens junctions), or radius less than r_{crit} are not permitted to pass the checkpoint and remain indefinitely in G0 (become quiescent). The first of these rules represents the concept of contact inhibition of proliferation, whereby cells with multiple adherens junctions leave the proliferative pool, and the second represents the requirement for cells to attach and spread on the substrate in order to proliferate. The choice of the parameters n as a threshold bond number ($n = 4$ for monolayer culture simulations), and r_{crit} as a threshold minimum radius ($r_{crit} = 15 \mu\text{m}$) is essentially arbitrary, but has been demonstrated to produce results that give qualitative agreement with experimental data (Walker et al., 2004b).

It is known that signalling via the EGFR and MAPK intracellular pathway plays a critical role in the transcription of the cyclin D1 gene, hence allowing cells to pass the G1 restriction point and enter S phase. Our goal was to devise a mathematical model of autocrine/homotypic paracrine EGFR signalling, and to incorporate this into the existing *Epitheliome* agent framework by modifying the existing checkpoint rules. This is a critical step in introducing a molecular-level ‘mechanism’ into a currently phenomenological model, and will form the first link between molecular-level events and growth on a tissue length scale.

The agent specification was extended to allow the user to specify an autocrine, or a particular concentration of exogenous EGFR ligand to be included in the simulation. An initial population of agents is then created, containing additional memory parameters including the total number of EGFR (up to a maximum number of 1×10^5 depending on cell cycle position of the agent at the start of the simulation, Waters et al., 1990) and ratio of surface to internal receptors (in the absence of the relevant data, a random number up to a third of the total number are initially defined as internal). Additionally, the agent will contain information about the rate of ligand release, and numbers of any surface or internal ligand–receptor complexes defined in the model (initially set to zero). While in the G1 phase, every agent will incrementally increase its total free receptor number in such a way that the surface density of receptors at the end of the growth phase is identical to that immediately after mitosis. The agent rules and correction for overlap are then executed for a single agent time step, Δt_a representing 30 min, as illustrated in Fig. 1, and the mathematical signalling model is solved for a period also representing a total of 30 min, as described below.

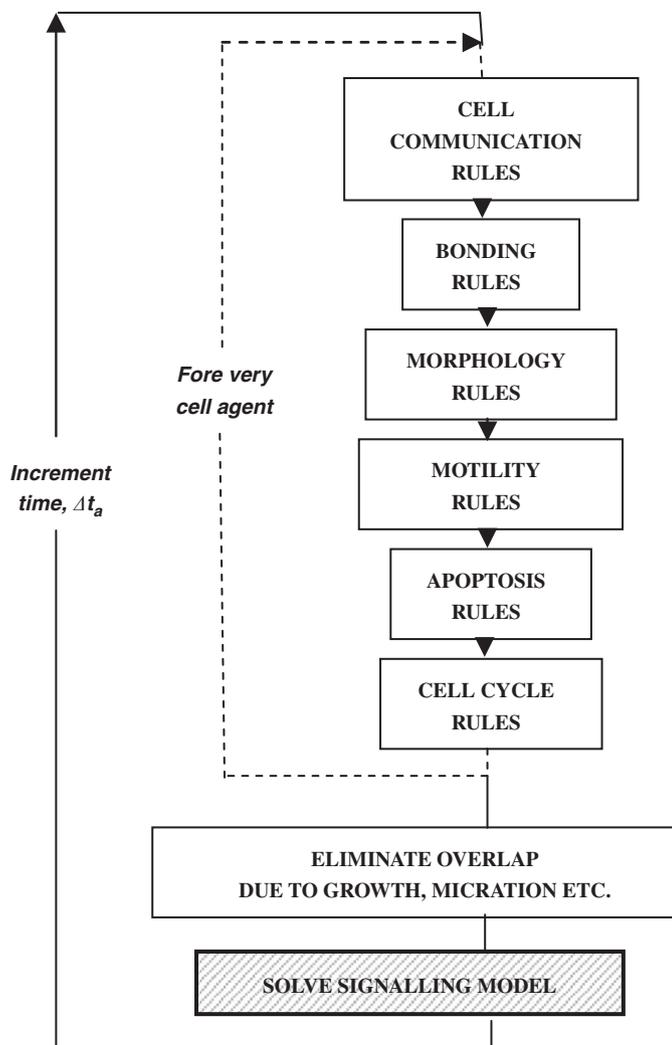


Fig. 1. Schematic of model solution process.

2.2. Mathematical model of intercellular signalling

This model can be considered as an assembly of equations representing a number of aspects of autocrine signalling: (a) ligand release and diffusion, (b) ligand–receptor binding, and (c) activated receptor trafficking and recycling. The system of equations is constructed on a grid system, with the x – y plane parallel to the cell monolayer, and the z plane representing the medium above the cell surface. This representation is valid as urothelial cells, on which our computational model is based, grow in monolayers in culture and do not form three-dimensional structures unless specifically induced to do so (Cross et al., 2005). The grid spacing in the x and y directions is similar to the minimum agent radius (10 μm) but is variable in the z dimension. At the end of each agent iteration, the current agent positions are interpolated onto the $z = 0$ plane of the three-dimensional grid, with a minimum of four nodes corresponding to each agent. Although the agents are three dimensional as they have both a surface area in the x – y plane and height in the z plane, it is assumed that ligand is released at the same z location (i.e. $z = 0$), irrespective of agent height. This simplification is justified as urothelial cells in culture assume a spread morphology immediately after plating, where the cell height is significantly less than the length.

A set of partial differential equations, representing ligand diffusion are assembled based on this discrete representation of the cell culture/medium system, whereas ligand–receptor binding and trafficking is represented by a set of ODEs. As the release and diffusion of ligand takes place, a concentration of free ligand will be associated with every node in the model, but nodes associated with agents will also represent concentrations of membrane-bound receptors and receptor–ligand complexes, and internal receptors and complexes. As these species occupy three distinct compartments—extracellular medium, cell membrane and cytosol, concentrations of species are calculated differently depending on the compartment occupied. Extracellular species are converted from numbers of molecules released per unit time to nanomolar concentrations using the volume represented by each node. Intracellular species are converted to nanomolar concentrations using the volume of the cell agent with which they are associated (assuming that 70% of this total volume is composed of water, within which homogenous mixing of species can occur). Membrane-bound species are scaled to extracellular concentrations when calculating reactions that take place on the surface of the cell, and intracellular volumes when considering trafficking processes. This is a similar approach to that used by Kholodenko et al. (1999).

A mass action kinetics scheme has been used to construct the ODEs that represent ligand–receptor binding and trafficking. Standard mass action kinetics have been shown to be limited in their ability to accurately represent reactions that are confined to two-dimensional surfaces such as biological membranes (Berry, 2002). However, all

kinetic parameters reported in the literature for ligand–receptor binding have been derived using this scheme, and thus it has been applied in this model.

An explicit (forward difference) finite difference equation is constructed to represent the diffusion of soluble ligand between nodes, and ODEs are used to represent the concentrations of other chemical species (surface and internal free receptors and receptor–ligand complexes) due to ligand binding and receptor and complex internalization and trafficking. Details of the equations used are given in Appendix A. This explicit approach to reaction diffusion problems is stable only if the time step dt is smaller than the characteristic time of the fastest process modelled. In particular, for the solution of a 3D diffusion problem:

$$dt \leq \frac{inc^2}{6.D},$$

where inc is the smallest spacing between adjacent nodes. A time step of $dt = 0.002$ min was used, and it was demonstrated that no significant change in results at the single cell level was obtained by further reduction of this time step. Results were also compared with those obtained using an implicit (backward difference) scheme for the diffusion calculations. As it is the concentration of free ligand at the cell surface at any point in time that will determine binding, whereas the concentration at any other point in the medium is relatively unimportant, a larger nodal separation was used in the z direction than in the x – y plane. Testing demonstrated that results were unaffected as long as at least 20 nodal increments were used in this plane (data not shown).

The values of the various parameters used are given in Table 1. All ligand-related parameters are based on those in the literature for EGF. Note we have assumed that $f_{rec} + f_{deg} = 1$, and that the degradation–recycling ratio and rate constants are identical for free receptors and complexes in endosomes (DeWitt et al., 2001). We have also assumed that there is no reduction of ligand concentration in the extracellular medium due to, for instance, sequestration by binding to extracellular matrix proteins. We have not explicitly modelled receptor dimerization, a process which we have assumed to be instantaneous.

The solution scheme for the signalling model is summarized in Fig. 2. After the numerical solution described above is run for a period equivalent to an agent time step of 30 min, the agents are updated with the final concentrations of the various compartmental chemical species calculated at the appropriate node. The nodal values of exogenous ligand concentrations are saved to form the initial conditions for the next signalling solution.

2.3. Feedback into agent model

It is known that signalling via the EGFR receptor can modulate a number of aspects of cell behaviour including

Table 1
Parameters used in signalling model

Constant	Description	Value	Units	References
L_{rel}	Rate of ligand cleavage	500	min^{-1}	DeWitt et al. (2001), Oehrtman et al. (1998), Shvartsman et al. (2002), Waters et al. (1990)
D	Ligand diffusion constant	6000	$\mu\text{m}^2 \text{min}^{-1}$	Shvartsman et al. (2002)
nR	No. of surface receptors per cell	1×10^5		Oehrtman et al. (1998), Shvartsman et al. (2002)
R_{syn}	Rate of free surface receptor synthesis	300	min^{-1}	DeWitt et al. (2001)
k_a	Receptor–ligand association constant	0.07	$\text{nM}^{-1} \text{min}^{-1}$	DeWitt et al. (2001), Maly et al. (2004), French et al. (1995), Lenferink et al. (2000)
k_d	Dissociation constant	0.23	min^{-1}	DeWitt et al. (2001), Maly et al. (2004), French et al. (1995), Lenferink et al. (2000)
k_e	Complex internalization rate constant	0.2	min^{-1}	Schoeberl et al. (2002), Oehrtman et al. (1998), Pribyl et al. (2003), Wiley et al. (1991)
k_{er}	Free receptor internalization rate constant	0.03	min^{-1}	Oehrtman et al. (1998), Starbuck and Da (1992), Shvartsman et al. (2002) Wiley et al. (1991)
k_{deg}	Internal complex/receptor degradation rate	0.022	min^{-1}	DeWitt et al. (2001)
k_{rec}	Internal complex/receptor recycling rate	0.058	min^{-1}	DeWitt et al. (2001)
f_{deg}	Fraction of internal receptors/complexes degraded	0.45	min^{-1}	DeWitt et al. (2001)

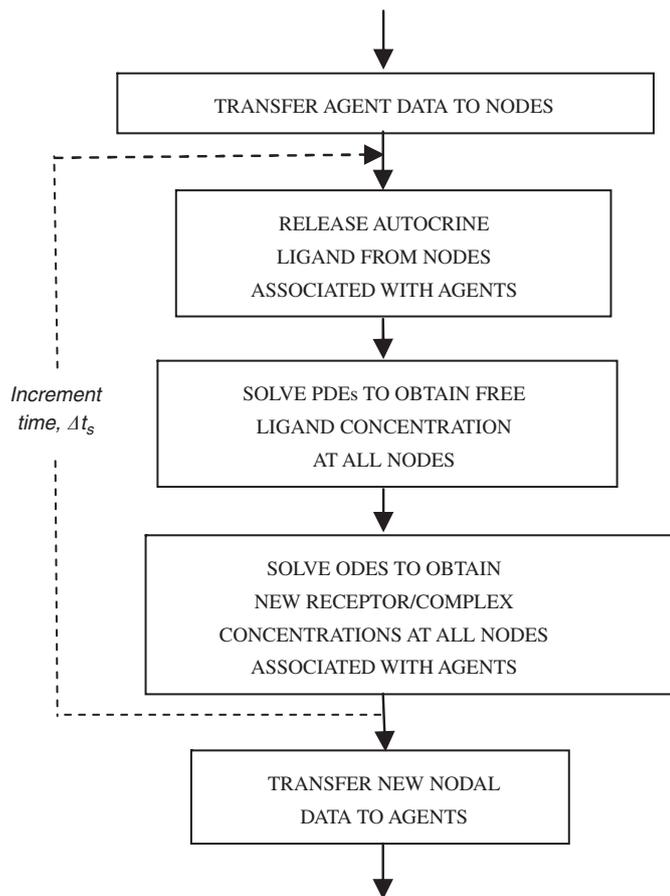


Fig. 2. Schematic of signalling model solution.

proliferation, migration, differentiation and also influence receptor expression and ligand release rate, thus forming positive and negative feedback loops. In the current model, we have considered only the effect of receptor occupancy on the decision of individual cells whether or not to proliferate, or become quiescent. This decision is made at

the existing G1–G0 checkpoint approximately half way through the G1 phase, which is traversed by every proliferating cell once per cell cycle. This new ‘rule’ is used in addition to the existing rules of the *Epitheliome* model relating to contact inhibition and cell morphology. The rule is stochastic, in that it involves calculation of a probability that the cell will continue in G1 based on the level of receptor occupation, i.e. for the i th cell:

If ($cell\{i\}$ has bonds with less than 4 neighbouring cells)
AND ($cell\{i\}_{radius} > 15\ \mu\text{m}$)
Probability of passing checkpoint
 $P_{pass} = cell\{i\}_{fraction_total_receptors_occupied} \times 4$
IF $P_{pass} > \text{random number}$
 $cell\{i\}$ passes checkpoint
ELSE
 $cell\{i\}$ enters G0

Hence a cell with 25% or more of its total receptors occupied by ligand will definitely continue cycling (assuming no contact inhibition and a sufficient spread morphology). This figure was based on the work of Knauer et al. (1984), who observed a maximum proliferation rate in fibroblasts for 25% total receptor occupation. At this stage, we have not discriminated between surface complexes and those internalized in endosomes, assuming both to contribute to the pro-proliferative signal, as suggested by Haugh et al. (1999).

When a cell rests in G0, it is permitted to re-enter the cell cycle only if all the necessary conditions are fulfilled (i.e. contact with substrate is sufficient, fewer than four intercellular bonds and at least 25% of total receptor number is occupied). Re-entry into the G1 phase is deterministic—there is no dependence on random numbers; this is an important property of the model that has allowed it to be applied to regenerative wound repair

processes, as removal of intercellular bonds by wounding allows cells to re-enter the cell cycle (Walker et al., 2004a).

This integrated agent-signalling model was used to explore the effect of a number of cell/environment-related parameters on the autocrine/homotypic paracrine signalling processes of a population of cells in terms of predictive effect on population growth. In particular, we have examined the effect of (a) seeding cell density, (b) exogenous EGF concentration, (c) volume of medium added to the cell culture (represented by height in the z direction in the diffusion/binding model), and (d) exogenous calcium concentration on receptor occupancy and population expansion. A sensitivity analysis was also carried out in order to identify the parameters most likely to affect the signalling behaviour of the system. All simulations were carried out using the Matlab software package (The Mathworks Inc., www.mathworks.com).

3. Results

3.1. Effect of seeding cell density

In order to determine the effect of increasing the size of the starting population seeded onto a culture dish of constant surface area, agent simulations were initiated with 25, 50 or 125 cell agents seeded randomly onto a $500 \times 500 \mu\text{m}^2$ substrate (equivalent to seeding densities of 100, 200 and 500 cells per mm^2 or 1×10^4 , 2×10^4 and 5×10^4 cells per cm^2 , respectively). Simulations were run as described above for 100 iterations, representing 50 h or approximately 2 days. Unless stated otherwise, exogenous calcium concentrations were set to 0.05 mM, resulting in minimal bonding between agents. Simulations were repeated for a number of values for the initial concentration of exogenous ligand, representing EGF added to the culture medium, and total medium volumes. For each set of initial parameters, a total of three simulations with different starting configurations of cell agents and random seeds were run, in order to eliminate possible artefacts arising from the stochastic nature of the model. At the end of each iteration, parameters describing the number of free and occupied surface and internal receptors associated with each agent were recorded.

The predicted effect of seeded cell density on (a) the mean fraction of receptors that are occupied for each cell, (b) the mean concentration of free ligand in the culture medium and (c) the rate of population growth (defined as total cell number at time t divided by seeded cell number) for a simulation with no added growth factors and a constant culture medium height of 1.0 mm is shown in Fig. 3. The results shown are the mean results for the three simulations carried out for this parameter set. For clarity, results are plotted every 3 h and error bars representing the standard deviation obtained from the three simulations run with identical starting parameter sets are included to provide an idea of the degree of stochastic variation.

In this case, EGFR activation associated with any agent in the system will be due to exogenous ligand secreted by that particular agent (autocrine) or other agents in the vicinity (homotypic paracrine effect). The model clearly predicts a correlation between seeded cell density and the rate of receptor occupation and accumulation of free ligand in the medium. Initially, the population growth rate is similar for all three cell densities, simply because the cell agents were created with a random distribution throughout the G1, S and G2 phases of the cell cycle, so a certain proportion of cells in each simulation will have already passed the checkpoint and will thus be committed to at least one division. The results suggest that as cells reach the G1 checkpoint, no cell will have 25% receptor occupancy, and according to the agent rules we have defined, none will be guaranteed to continue in the cell cycle. However, as we have defined the probability of passing the checkpoint as linearly dependent on receptor occupancy, agents in the higher cell density simulations will have more receptors occupied, and hence be more likely to continue to proliferate. In the case of the two lower cell density simulations, the smaller agent numbers results in a more significant stochastic effect, and there is no difference between the growth rates. None of the models produced a confluent monolayer within the time period of the simulation.

Fig. 4 shows results for the case where 1.0 nM EGF is added at the start of the simulation, but is not replenished. In this case, mean receptor occupancy of all agents very quickly reaches the critical 25% level, and all agents will pass the G1 checkpoint, resulting in similar rates of population growth in all cases. However, because the growth factor is not replenished, but is internalized and broken down by the cells at a greater rate than endogenous ligand can be produced, the concentration of EGF in the medium begins to fall. The greater the initial cell number, the higher the consumption rate and hence receptor occupancy declines more quickly in the higher seeded cell density simulations. After approximately 24 h, the most densely seeded model approaches confluence and population growth slows significantly. Eventually, stabilization of the cell population leads to a gradual increase in secreted exogenous EGF concentration. The 200 cells mm^{-2} model reaches confluence at the end of the simulation period, whereas the model with the lowest seeded cell density remains subconfluent throughout the simulation.

3.2. Effect of exogenous EGF concentration

Results for simulations initialized with 50 cells agents (200 cells mm^{-2}), a medium height of 1.0 mm and EGF concentrations of 0, 0.1, 0.5 and 1.0 nM are shown in Fig. 5. EGF in the model is not replenished during the simulation.

Inspection of Fig. 5a suggests that only the highest concentration of EGF is sufficient to give an initial receptor occupancy of 25%, though this level will no

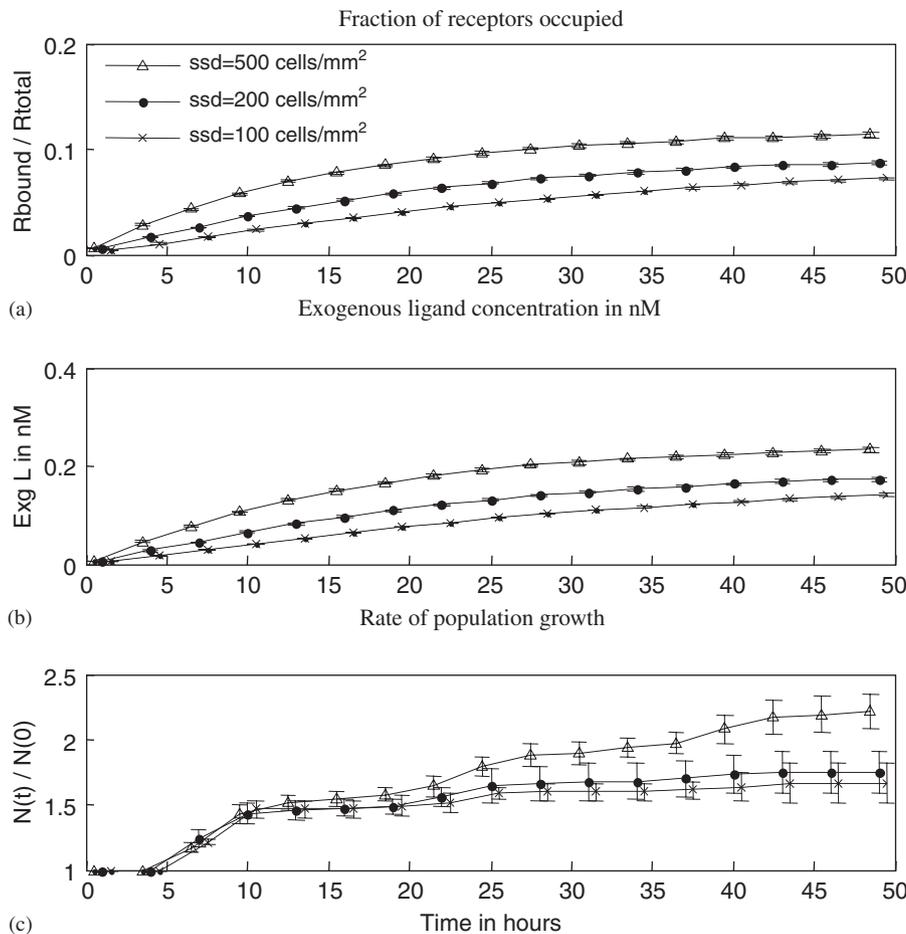


Fig. 3. Model results showing effect of seeded cell density on signalling and growth for endogenously produced ligand only: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth for autocrine ligand production only (no exogenous EGF, medium height = 1.0 mm, exogenous calcium concentration = 0.05 mM).

longer be attained after a day if the growth factor is not replenished (Fig. 5b). EGF is also consumed faster than it is produced at an initial concentration of 0.5 nM, but in the 0.1 nM case, autocrine ligand cleavage exceeds consumption. These results suggest that ligand consumption and production should be close to equilibrium for this particular seeded cell number for an initial EGF concentration of approximately 0.2 nM. As expected, population growth rate is related to the initial EGF concentration, with initial levels of 1 and 0.5 nM significantly increasing the growth rate above that predicted for the endogenous ligand-only case. Adding a very low quantity of EGF to the medium (0.1 nM) does not have an effect on the growth rate as the molecules quickly become bound to surface receptors, internalized and degraded.

3.3. Effect of culture medium volume

Fig. 6 shows the results for simulations initialized with 50 cells agents (200 cells mm⁻²), with no added EGF and medium heights of 1.0, 1.5 and 3.0 mm (in a 3 cm diameter Petri dish, equivalent to approximately 0.7, 1.1 and 2.1 ml, respectively). It is assumed there is no evaporation or net

water consumption by cells, so medium height will remain constant throughout the simulation. In this case, receptor binding will be due to endogenous EGF only, and as shown in Fig. 6a, initial receptor occupancy rates are very low. However, as expected, for similar rates of ligand cleavage, the concentration of EGF in the medium will increase more quickly for smaller medium volumes (Fig. 6b). However, as these concentrations remain very low throughout the simulation (<0.2 nM in all cases), there is no effect on population growth rate.

The results for a similar set of simulations, but with the initial EGF concentration set to 1.0 nM, are shown in Fig. 7. In this case, EGF is depleted from the medium more quickly for smaller medium volumes, resulting in a significant reduction in receptor occupancy and a decline in population growth rate for a medium height of 1 mm.

3.4. Effect of exogenous calcium concentration

Increasing the calcium concentration in the extracellular medium of our model will not have a direct effect on EGFR signalling, but may have an indirect effect by increasing the tendency of cell agents to group together in

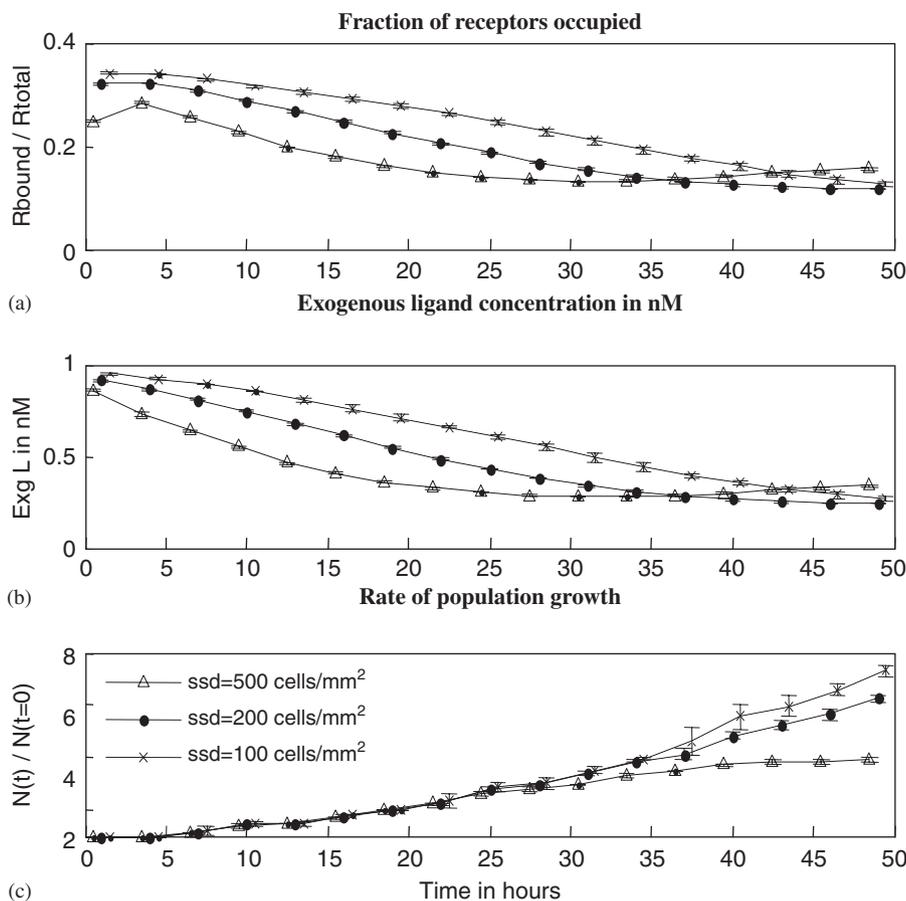


Fig. 4. Model results showing effect of seeded cell density on signalling and growth due to endogenous and exogenous ligand: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth (1 nM exogenous EGF, medium height = 1.0 mm, exogenous calcium concentration = 0.05 mM).

colonies, rather than remaining dispersed throughout the model. Similar starting configurations of cell agents were used as for the low calcium simulations ($[Ca^{2+}] = 0.05$ mM), but the exogenous calcium level was fixed at 2 mM (close to physiological levels), the EGF concentration at 0 nM, and the medium height at 1.5 mm. The distribution of agents after 20 iterations (10 h) simulation in 0.05 and 2.0 mM exogenous calcium is shown in Fig. 8, superimposed onto a surface plot of the distribution of exogenous EGF on the $z = 0$ plane. It can be seen that the concentration of EGF close to cell colonies in the higher calcium-containing medium appears to be higher, and there is a gradient across the surface in the lower calcium model, with higher EGF concentration associated with the region of highest cell density in the bottom left corner. However, these plots have been scaled to emphasise differences in EGF concentration, and the ratio between maximum and minimum levels shown are typically of the order of 5–10%.

A more quantitative representation of the results is shown in Fig. 9. For clarity, results for only the intermediate seeding density of 200 cells mm⁻² are shown. It can be seen that after approximately 20–25 h, there is an increase in growth rate in the lower calcium simulations relative to those in higher calcium, although there is no

difference in the level of secreted EGF in the medium or receptor occupancy. This difference in population growth rate can be attributed to the contact inhibition rules in the agent model that state that agents with four or more intercellular bonds enter the G0 phase, and are thus, at least temporarily, prevented from completing that round of cell division. As the agents move and form bonds stochastically, there is a significant degree of variation between replicate simulations, as demonstrated by the large error bars. Movies showing the growth of the agent populations in the two calcium concentrations are available in the supplementary material provided online (http://www.sciencedirect.com/* *...).

These results suggest that the difference in growth patterns observed in low and physiological calcium environments does not have a significant effect on the pattern of ligand diffusion, and hence binding to EGFR on neighbouring cells.

3.5. Sensitivity analysis

The sensitivity of a model to its constituent parameters can be assessed by calculating the fractional change in the value of a designated output parameter in relation to the

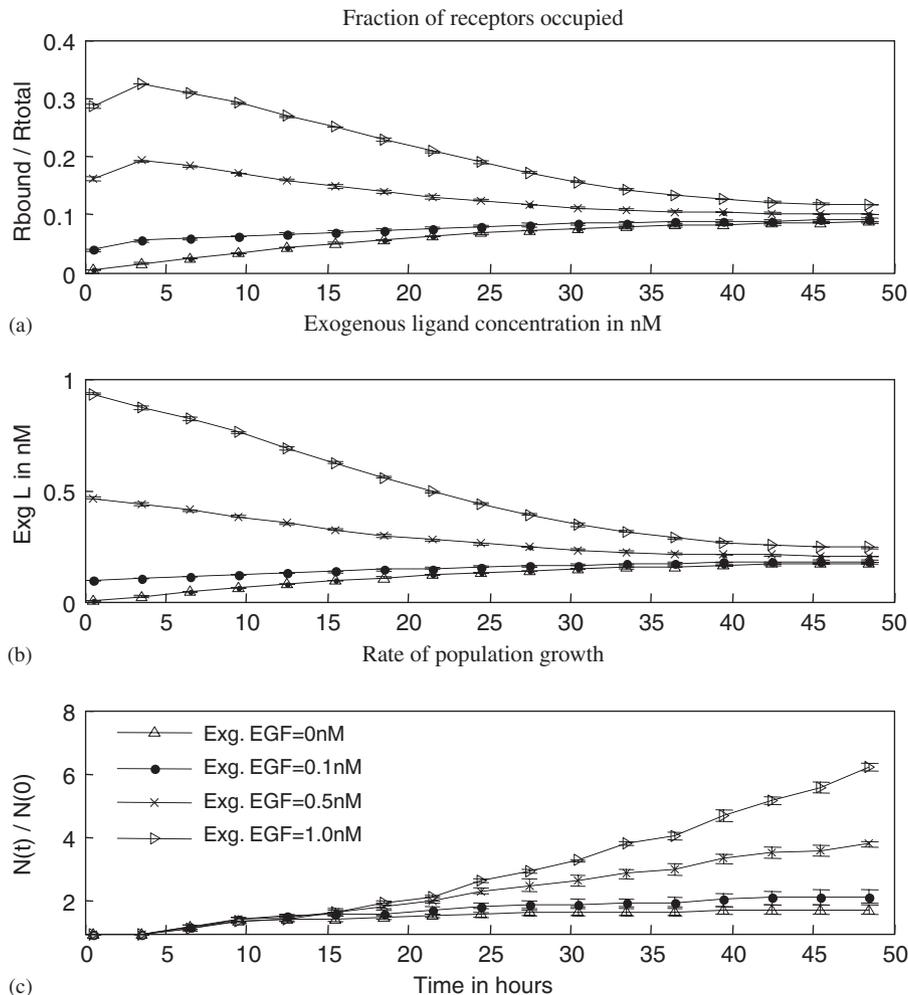


Fig. 5. Effect of exogenous EGF concentration on: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth (cell seeding density = 200 cells mm⁻², medium height = 1.0 mm, exogenous calcium concentration = 0.05 mM).

fractional change in a particular input parameter, when the value of all other inputs in the set are held constant. In order to investigate the sensitivity of our signalling model to the kinetic components defining ligand release, diffusion, binding and trafficking, we used a model with medium height 1 mm, zero exogenous EGF and a population of 50 cell agents. The output parameter of interest was defined to be the mean receptor occupancy, RO , across the cell population after 30 min of simulation, i.e:

$$\overline{RO} = \frac{\sum_{n=1}^N (C_s + C_e) / (C_s + C_e + R_s + R_e)}{N},$$

where C_s and C_e are the number of surface and endosomal complexes, and R_s and R_e the number of surface and endosomal free receptors, respectively, and the summation is over all nodes in the signalling model that are associated with cells. The sensitivity S , of RO to each input parameter, ip , was defined as the ratio of the fractional change in RO to the fractional change in ip :

$$S = \frac{\Delta RO}{\Delta ip},$$

where

$$\Delta RO = \frac{RO_0 - RO_1}{RO_0} \quad \text{and} \quad \Delta ip = \frac{ip_0 - ip_1}{ip_0}.$$

RO_0 indicates the mean receptor occupancy obtained for the input value quoted in Table 1 (ip_0) and RO_1 the value obtained for the perturbed input value (ip_1). A negative value of S indicates that an increase in the input parameter causes a reduction in RO . A value of ± 1 indicates that a 100% change in ip will cause a 100% change in RO , whereas $S = 0$ indicates that the receptor occupancy is completely non-dependent on the input variable.

S was assessed by both doubling and halving each of the input parameter values given in Table 1, and the mean of the two values of S for each ip are plotted in Fig. 10. It is apparent that receptor occupancy, and by extrapolation, cell proliferation is highly dependent on ligand release rate, L_{clv} , with a high degree of dependence on receptor–ligand association constant, k_a ligand diffusivity D and dissociation constant k_d . The total receptor number and internalization and trafficking rates for receptors and complexes have a considerably smaller effect on receptor occupancy.

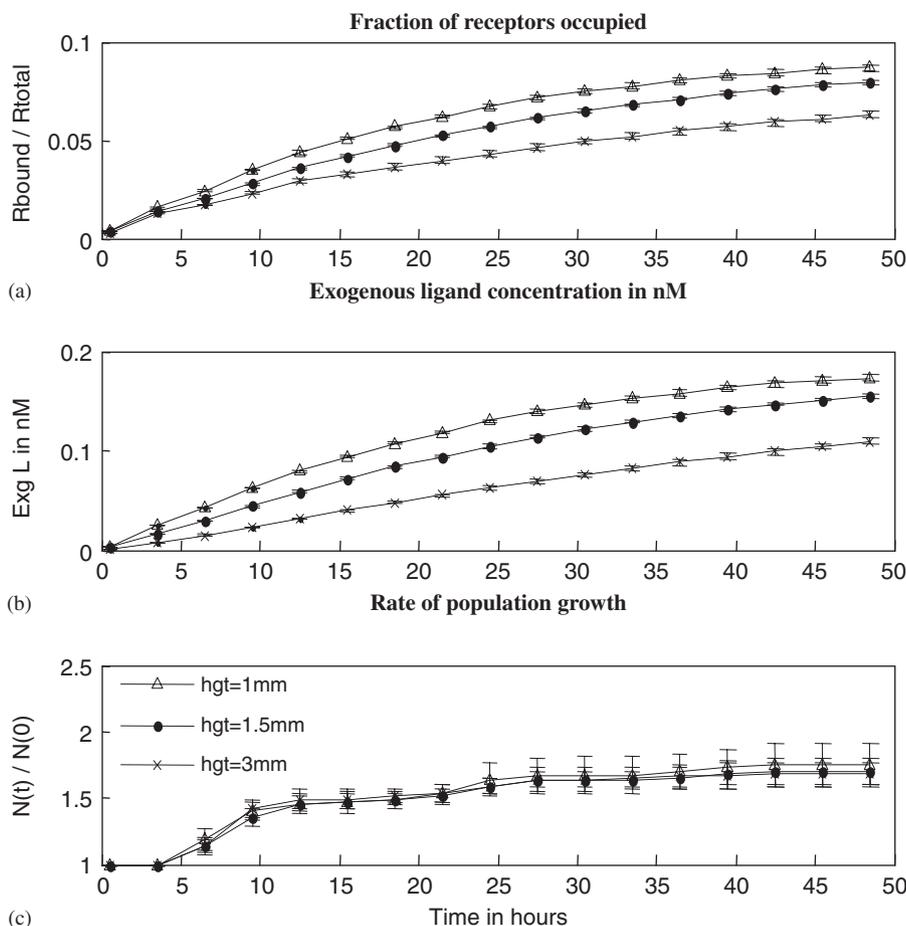


Fig. 6. Effect of culture medium volume on signalling and growth for endogenously produced ligand only: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth (cell seeding density = $200 \text{ cells mm}^{-2}$, no exogenous EGF, exogenous calcium concentration = 0.05 mM).

4. Discussion

Signalling through the EGFR is known to be vital in controlling cell proliferation in a number of tissue types. Previous mathematical models have been developed to describe this and other receptor–ligand systems at a cellular level. By combining a mathematical model of molecular binding and diffusion with our previously developed software agent representation of biological cells, we have taken this concept a step further and forged a link between the sub- and supra-cellular levels of the computational model. This multi-scale model, which is capable of representing both cellular- and molecular-level behaviour in real time, without the use of look-up tables when switching between scales, is a powerful tool for exploring the complex feedback relationship between environment, signalling and response. The main example that we have explored here is the feedback relationship between cell density and production and binding of autocrine/homotypic paracrine EGFR ligands, and the subsequent effect on cell population growth.

In the absence of exogenous EGF, the model predictions support the intuitive expectation that the amount of EGF ligand released into the extracellular milieu is dependent on

the cell density, and the concentration of exogenous ligand increases most rapidly in cultures bathed in a small volume of medium (Fig. 6), which leads to a greater degree of receptor occupancy throughout the entire cell population, and ultimately an increased population growth rate. Likewise, Fig. 5 demonstrates that adding higher concentrations of growth factor to the culture medium should result in higher receptor occupancy and faster population growth.

An alternative modelling approach could involve replacing the molecular diffusion, binding and trafficking calculations with simple agent-based rules (e.g. cell density = X , so probability of passing the G1/G0 checkpoint = $Y \dots$). This would have the advantage of reducing simulation time, but would fail to capture the concept of cells sensing only their immediate environment by virtue of molecular interactions at the cell surface. Explicit inclusion of the mathematical model allows us to represent the signalling system in a quantitative way, at a level of detail which simple agent rules would fail to capture. The fine balance between ligand secretion and consumption for different exogenous EGF levels, cell populations and medium volumes would be impossible to explore with cell agents alone.

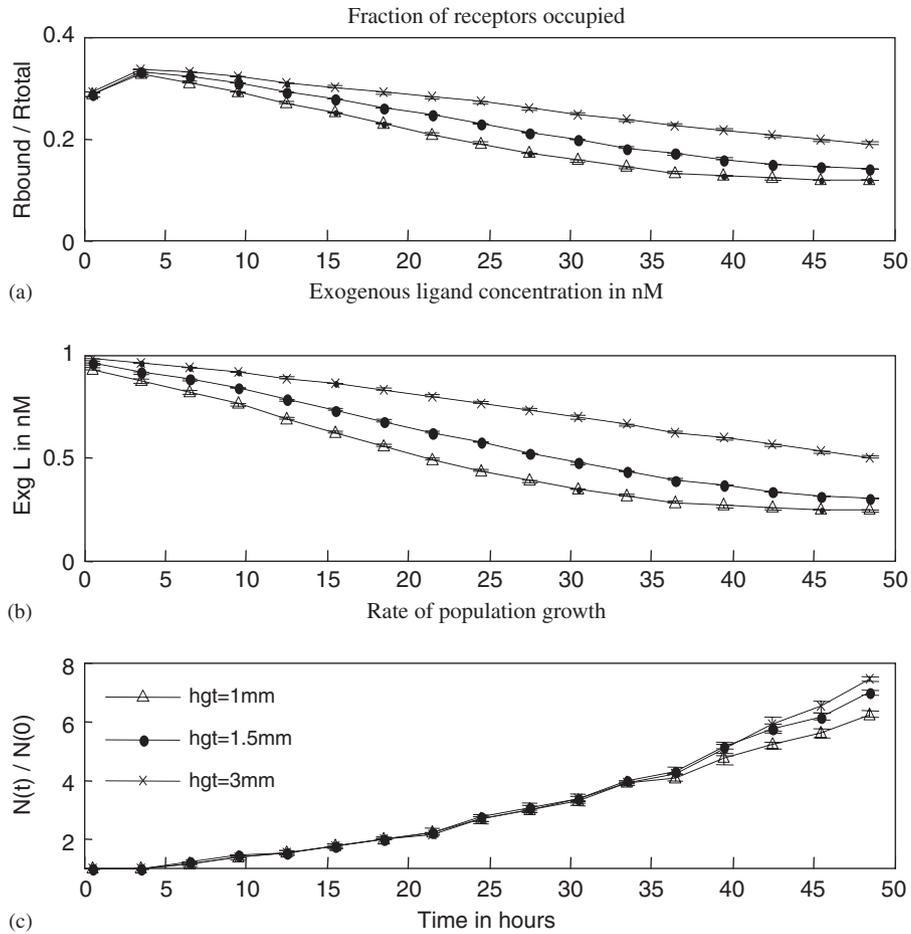


Fig. 7. Effect of culture medium volume on signalling and growth for exogenous ligand: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth (cell seeding density = $200 \text{ cells mm}^{-2}$, 1 nM exogenous EGF, exogenous calcium concentration = 0.05 mM).

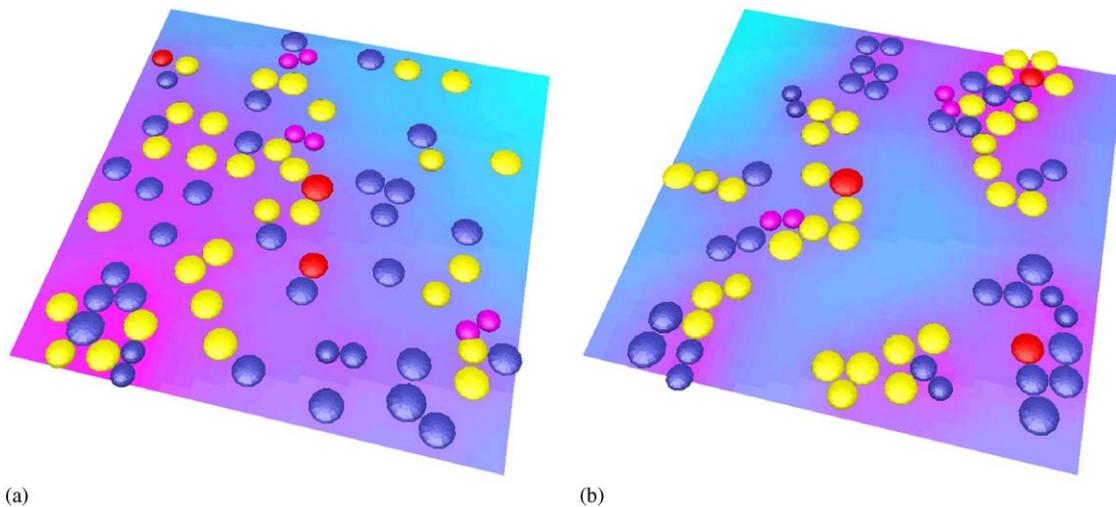


Fig. 8. Plots showing simulated concentration of secreted EGF for cell agents in (a) 0.05 mM and (b) 2.0 mM exogenous calcium after 10 h. Starting cell density = $200 \text{ cells mm}^{-2}$, medium height = 1.5 mm and no exogenous EGF added in either case. Agent colour key: red = stem cell, blue = transit amplifying cell, pink = cell in M phase, yellow = cell in G0 phase.

By adjusting the exogenous calcium level defined in our model, we have also explored the interplay between the cell environment and signalling via endogenous soluble mes-

sengers. It is well known that the effect of raising extracellular $[\text{Ca}^{2+}]$ from low to physiological levels is to increase the tendency of cells to form adherens junctions

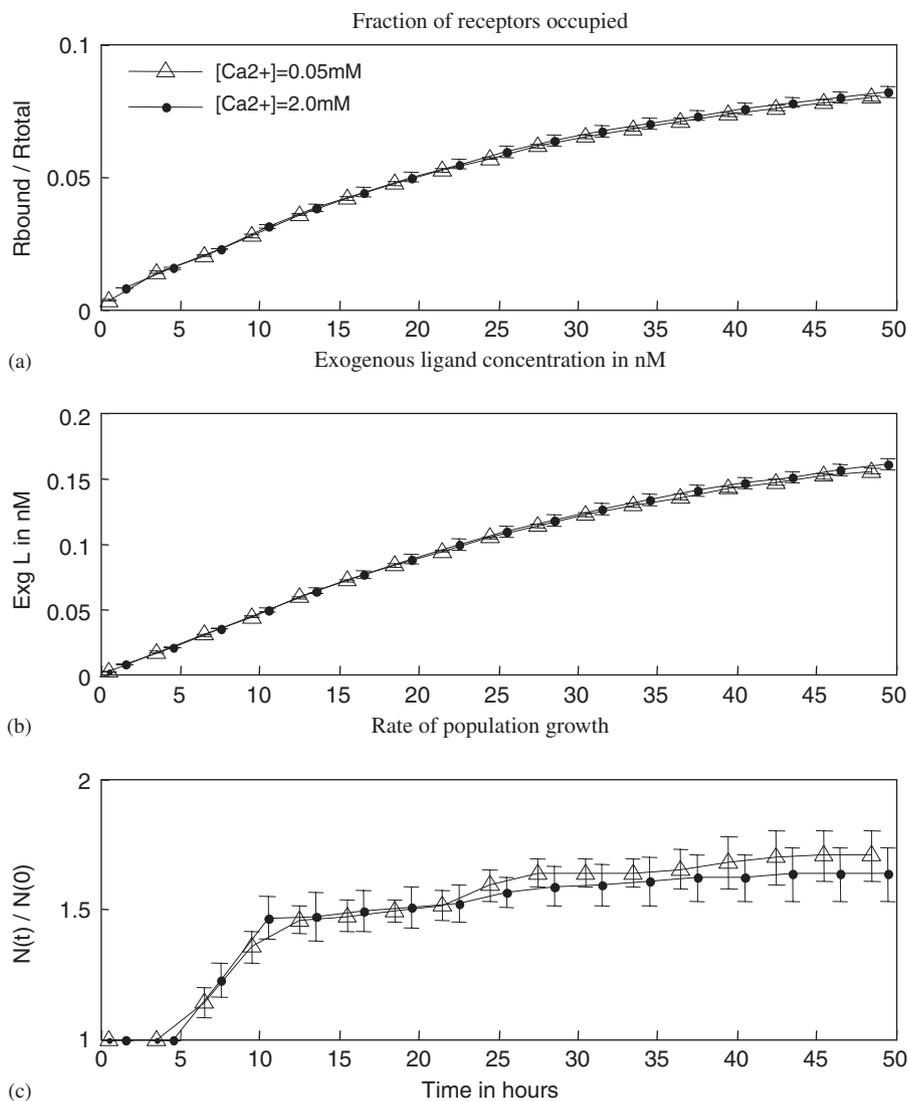


Fig. 9. Effect of exogenous calcium concentration on signalling and growth for endogenously produced ligand only: (a) mean fraction of receptors occupied, (b) mean concentration of free ligand in the culture medium, and (c) rate of population growth for autocrine ligand production only (seeded cell density = 200 cells mm⁻², no exogenous EGF, medium height = 1.5 mm).

via homotypic E-Cadherin interactions, and hence grow in colonies, rather than retain a more migratory phenotype and remain evenly dispersed over the culture substrate. We have previously explored the relationship between calcium concentration and the pattern of tissue growth in both urothelial cells and keratinocytes (Walker et al., 2004b; Walker et al., in press). In the case where groups of ligand-secreting cells are growing in close contact with each other, it might be expected that there would be a higher net uptake of ligand by neighbouring cells, and less loss into the bulk medium, resulting in a higher degree of receptor occupancy in higher calcium cultures. The results of this model (Figs. 8 and 9) suggest that this is not actually the case. Examination of the dynamics of ligand release, binding and diffusion at a subcellular level shows that for the set of kinetic parameters used in these simulations, diffusion takes place at a much faster timescale than

ligand–receptor binding, and most ligand is lost into the medium and does not immediately bind to the receptors of the secreting cell—i.e. signalling is predominantly homotypic paracrine, rather than autocrine in nature. This was observed to be the case irrespective of the time step size or the order of solution (e.g. ligand diffusion before receptor–ligand binding, or vice versa). There is insufficient heterogeneity in the distribution of ligand in the culture medium to result in a significant heterogeneity in receptor activation, and hence similar patterns of ligand uptake are observed irrespective of the distribution of cells. This result supports the findings of Batsilas et al. (2003), who used a stochastic model to demonstrate that the probability of ligand recapture by the releasing cell is independent of local cell density, with signalling primarily of a paracrine nature in typical cell culture conditions. This suggests that we might be justified in eliminating the diffusion aspect of the

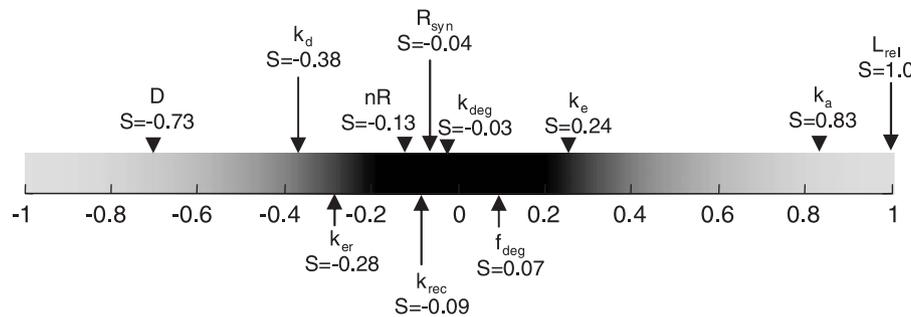


Fig. 10. Diagrammatic representation of results of sensitivity analysis. Sensitivity S is defined as the fractional change in the value of the designated output parameter in relation to the fractional change in a particular input parameter. The output parameter considered is the mean receptor occupancy per cell after 30 min simulation (no exogenous EGF, medium height = 1.0 mm, cell seeding density = 200 cells mm^{-2}). Input parameters are listed in Table 1.

solution from our model, and representing the process of ligand release and binding by a simpler set of ODEs and assuming the ligand concentration to be homogeneous throughout the medium. Alternatively, we could use a boundary homogenization method similar to that employed by Batsilas et al. (2003) and Berezhkovskii et al. (2004), whereby the lower surface of the model is represented by an absorbing surface with an effective rate constant that depends on the mean receptor number, cell radius, binding affinity and cell density. This simplification would be at odds with the central concept of our modelling paradigm, which is the representation of cells as individual entities. Maintaining the inclusion of discrete cells will allow us to extend our model to examine the behaviour of heterogeneous populations of cells, for instance, those expressing different numbers of surface receptors, or exhibiting different rates of ligand release, or the capacity to release different ligands of the EGFR family (e.g. amphiregulin, TGF α , heparin-binding EGF). However, in cases where these scenarios are not of interest, such simplification techniques would be a legitimate method of increasing efficiency. It should be noted that the model we present here does not currently have the capacity to represent heterogeneous distributions of receptors and ligand cleavage sites over the membrane surface, although this might be expected to influence the probability of a cell recapturing ligand cleaved from its surface.

The major limitation of any mathematical or computational model is that the simulations are only as accurate, or appropriate, as the choice of parameters used. This is an issue for modelling of EGFR–ligand interactions where the limited experimental data available have been obtained almost exclusively for EGF (though some data for TGF α is available), using a limited number of cell types, in particular, human and mouse fibroblasts. Our agent model, *Epitheliome*, has been developed to represent human epithelial cell types, which may exhibit quite different behaviour in terms of, for instance, receptor expression, internalization and degradation. None of the relevant kinetic parameters have been measured in epithelial cells, but sensitivity testing with our mathematical model demonstrates that doubling the rate of ligand release will

double the ratio of bound to free receptors after 30 min simulations (Fig. 10). Varying the association constant, k_a , ligand diffusivity D , or dissociation constant k_d will also have a significant effect on receptor occupancy, and by extrapolation, cell phenotype. Our results suggest that trafficking parameters, or the total receptor number will have a relatively small effect on the system. Greater sensitivity to ligand cleavage rate relative to free receptor availability is expected in a system where total receptor number is several orders of magnitude greater than free ligand. Very little experimental data are available for ligand cleavage rates in autocrine systems and the small number of values obtained experimentally (again, all using fibroblasts) show considerable variation. The problem is further compounded by the existence of potential feedback loops in the system, whereby activation of the EGFR can upregulate ligand and receptor expression (Clark et al., 1985; Coffey et al., 1987)—a phenomenon that is not incorporated into our current model, but could be included in the future.

Analysis of the sensitivity of the model to its constituent parameters also provides an insight into possible aberrant behaviour of the biological system. The role of the EGFR–ligand system in malignancy has long been acknowledged (as reviewed in (Normanno, 2006 #324)), and in certain tissue types, autocrine EGFR signalling has been implicated. For instance, the overexpression of the autocrine EGFR ligands EGF, TGF α and amphiregulin (AR) have been linked to various types of breast tumours (extensively reviewed by (Salomon, 1995 #325)). This fits well with our observation that in an autocrine ligand limited system, cell response will be extremely sensitive to ligand availability. Receptor over-expression and mutations which may potentially affect EGFR activity are other malignancy-related changes that could be probed with such a model, and further development could potentially result in a useful tool that could be employed in cancer therapy development.

Our link between the molecular- and cellular-level models relies on an observation that a population of fibroblasts responds in a particular way to a certain level of receptor occupancy (Knauer et al., 1984). Epithelial cells

will almost certainly exhibit different behaviour, both in terms of response to different EGFR-binding ligands and how the ligand is presented to the receptor (soluble or membrane-bound—see below), as well as to the level of receptor occupancy. For instance, it has been observed that the EGFR ligand amphiregulin, but not EGF itself is expressed by normal urothelial cells, and also that the effect of the addition of soluble growth factors to this cell type is to increase migration but inhibit proliferation. However, blocking EGFR activation inhibits proliferation, thus demonstrating that cells respond differently to endogenous and exogenous ligand (Varley et al., 2005). An important factor behind this observation is that endogenous ligand is believed to be expressed primarily in membrane-bound form, with either very low rates of proteolytic cleavage, or extremely fast uptake of any ligand released. Membrane-bound, or juxtacrine EGFR signalling has been described in a number of normal epithelial cell types, including skin (Tokumaru et al., 2000) and ovary (Pan et al., 2002), as well as being implicated in malignancy (Gerharz et al., 2000). Currently, we do not have access to the kinetic parameters for many of the ligands important for controlling phenotype in epithelial cells, and our model incorporates only the concept of the interaction of receptors with soluble, not membrane-bound growth factors. This, as well as the difficulties in making measurements on a sensitive auto-crine system where there are significant experimental uncertainties, for example, regarding the content of commercial tissue culture medium, suggests that it does not make sense to attempt a quantitative comparison between model predictions and experimental data at this stage. However, these current limitations should not be considered a reflection on the usefulness of the computational model, but simply highlight the requirement for a closely integrated iterative process of computational model development and in vitro experimentation in order to develop models that are both accurate and relevant to biologists.

In conclusion, although our current binding/diffusion model is quite simple, in combination with an agent-based model, it allows us to probe the complex feedback between subcellular molecular events, individual cell behaviour and patterns of tissue development that could not be achieved through differential-equation-based models alone. The model is extensible, so more complex trafficking dynamics could be incorporated, or intracellular signalling pathways, for instance, the MAP-kinase pathway, which is known to be critical in controlling cell proliferation, could be explicitly included. Ultimately, these could then be linked to other pathways—in particular, those mediated by integrin binding, which are known to interact with the EGFR–MAPK cascade to determine cell phenotype (Roovers and Assoian, 2000).

To date, we have focussed on the effect of EGFR signalling on the proliferative response of cells, but it has also been demonstrated that cell migration is also

promoted by receptor activation, and this concept could be incorporated into future models.

We are currently extending the mathematical model of EGFR–ligand binding to incorporate juxtacrine, as well as soluble ligand–receptor interactions. This is expected to provide a significant step in our ability to model the interactions of epithelial cells, and further our understanding of their role in the formation of normal, and eventually malignant, tissue structures.

Acknowledgements

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Appendix A

The diffusion of molecular species L , with diffusivity coefficient D in one dimension is given by

$$\frac{\partial L}{\partial t} = D \frac{\partial^2 L}{\partial z^2}, \quad (\text{A.1})$$

where t represents time, and z is the spatial coordinate. Extending this to three dimensions and rewriting using a forward difference (explicit) approximation for the concentration of a free ligand, L , this becomes:

$$\begin{aligned} \frac{L_{i,j,k}^{n+1} - L_{i,j,k}^n}{\Delta t} \approx & \frac{D}{\Delta x^2} \left[L_{i-1,j,k}^n - 2L_{i,j,k}^n + L_{i+1,j,k}^n \right] \\ & + \frac{D}{\Delta y^2} \left[L_{i,j-1,k}^n - 2L_{i,j,k}^n + L_{i,j+1,k}^n \right] \\ & + \frac{D}{\Delta z^2} \left[L_{i,j,k-1}^n - 2L_{i,j,k}^n + L_{i,j,k+1}^n \right], \end{aligned} \quad (\text{A.2})$$

where i, j and k represent the spatial grid coordinate in the x, y and z directions, respectively, $\Delta x, \Delta y$ and Δz are the node spacings in these directions, D is the ligand diffusivity, n indicates the current time step, and Δt is the time interval. In the case where $\Delta x = \Delta y$ (i.e. equal nodal spacings in the x – y plane), this can be rearranged to give

$$\begin{aligned} L_{i,j,k}^{n+1} = & L_{i,j,k}^n \left[1 - \frac{4D\Delta t}{\Delta x^2} - \frac{2D\Delta t}{\Delta z^2} \right] \\ & + \frac{D\Delta t}{\Delta x^2} \left[L_{i-1,j,k}^n + L_{i+1,j,k}^n + L_{i,j-1,k}^n + L_{i,j+1,k}^n \right] \\ & + \frac{D\Delta t}{\Delta z^2} \left[L_{i,j,k-1}^n + L_{i,j,k+1}^n \right]. \end{aligned} \quad (\text{A.3})$$

The contributions to the change in free ligand concentration due to ligand release, ligand–receptor dissociation and complex recycling can be written in ODE form. If the index m is substituted for the spatial indices i, j, k for clarity, the change in free ligand due to processes other than diffusion is given by

$$\frac{dL_m}{dt} = L_{rel,m} - k_a L_m R s_m + k_d C s_m + f_{rec} k_{rec} C e_m, \quad (\text{A.4})$$

where the first term on the right-hand side represents the rate of ligand release due to proteolytic cleavage, the second term represents binding of free ligand to free receptors, the third term, ligand released by the dissociation of bound receptors and the last term, ligand released from the recycling of bound receptors in endosomes. C_s and C_e are the concentration of bound ligand–receptor complexes on the cell surface and in endosomes, respectively, k_a is the ligand–receptor association constant, R_{s_m} is the concentration of free surface receptors, k_d is the ligand–receptor dissociation constant, f_{rec} is the fraction of internal complexes that are recycled and k_{rec} is the rate of complex recycling.

Similarly, the rate of change of free surface receptors can be written as

$$\frac{dR_{s_m}}{dt} = R_{syn,m} + k_d C_{s_m} - k_a L_m R_{s_m} + f_{rec} k_{rec} C_{e_m} - k_{er} R_{s_m}, \quad (A.5)$$

where R_{syn} is the rate of free surface receptor synthesis, the final term represents endocytosis of free receptors with rate k_{er} , and the other right-hand side terms are defined as above.

The rate of change of surface complexes is given by

$$\frac{dC_{s_m}}{dt} = -k_d C_{s_m} + k_a L_m R_{s_m} - k_e C_{s_m}, \quad (A.6)$$

where the final term represents the endocytosis of surface complexes at rate k_e . Similar expressions are formulated for the change in the concentration of internal receptors in endosomes:

$$\frac{dR_{e_m}}{dt} = k_{er} R_{s_m} - f_{rec} k_{rec} R_{e_m} - f_{deg} k_{deg} R_{e_m}, \quad (A.7)$$

where f_{deg} is the fraction of internal receptors degraded and k_{deg} is the rate of degradation. Note we have assumed that $f_{rec} + f_{deg} = 1$, and that the degradation–recycling ratio and rate constants are identical for free receptors and complexes in endosomes (DeWitt et al., 2001). Finally, the number of receptor–ligand complexes in endosomes is given by

$$\frac{dC_{e_m}}{dt} = k_e C_{s_m} - f_{rec} k_{rec} C_{e_m} - f_{deg} k_{deg} C_{e_m}. \quad (A.8)$$

The values of the various parameters used are given in Table 1 in the main text.

Appendix B. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtbi.2006.04.020.

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