Cell population modelling describes intrinsic heterogeneity: a case study for hematopoietic stem cells

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Abstract: The control of stem cell properties during in vitro expansion is of paramount importance for their clinical use. According to Food and Drug Administration (FDA) guidelines, phenotypic heterogeneity is a critical aspect influencing therapeutic response. Even if the authors ability to reduce heterogeneity were limited, the sources from which it arises should be well understood for safe clinical applications. The aim of this work was to describe theoretically the intrinsic cell population heterogeneity that is present even when cells are cultured in a perfectly homogeneous environment. A bivariate population balance model is developed to account for the heterogeneity in the number of receptors and receptor−ligand complexes per cell, and is coupled with a ligand conservation equation. As a case study, the model is applied to the hematopoietic stem cell expansion, considering the c-Kit receptor and stem cell factor pair. Results show the dependence of intrinsic heterogeneity from ligand concentration and the kinetics of its administration. By tracking the cell generations within the total population, the authors highlight intra- and an inter-generational contributions to total population heterogeneity. In terms of dimensionless variables, intrinsic heterogeneity is dependent on the ratio of the characteristic time of cell division to that needed by a newborn cell to reach its single-cell steady state.

1 Introduction

The therapeutic potential of stem cells is widely recognised [1], but their clinical use is still limited and one of the most critical aspects is the scarce number of cells available [2]. In vitro cell expansion has been proposed as a means to increase stem cell number, but requires a control of cell phenotypic properties at the end of the culture [3]. Also according to FDA guidelines, cell characterisation for safe clinical applications should involve the quantitative assessment not only of cell identity but also of heterogeneity in the cell population, for example, by monitoring cell surface antigens or biochemical markers [4]. In fact, distinct population subsets can give a different contribution once injected in vivo, making ambiguous the interpretation of the clinical response [5].

A cell culture environment where spatial inhomogeneities are present is a possible source of cell population heterogeneity [6]. In fact, cells exposed to different local culture conditions can produce different responses, for example, in terms of time for cell division or receptor expression [7]. Nonetheless, bioreactors are devices for performing cell cultures that, by convective motion, favour the dissipation of inhomogeneities in the medium and lead to the ideal case of perfect mixing, where all cells are exposed to the same local microenvironment [3, 8].

With this in mind, we aim at studying the sources of cell population heterogeneity in ideal systems where perfectly homogeneous cultures are performed. In this case, heterogeneity is intrinsic, related to cellular processes that produce variability of chemical and biochemical molecular species in different cell compartments, such as the membrane, cytoplasm or nucleus. We will focus on intrinsic heterogeneity on the cell membrane, as surface receptors mediate cell response to environmental stimuli, such as the presence of ligands [9], and as they are commonly used to experimentally characterise the stem cells, for example, by flow cytometry, a powerful technique that detects their distribution within the cell population [10].

A conceptual understanding of receptor-ligand dynamics mechanisms at the population level can be achieved by a computational approach, as a detailed understanding is precluded by the complexity of the population behaviour. Different mathematical formulations have been developed to describe stochastically or deterministically cell population properties [11]. Continuum models assume an average-cell behaviour, simulating a homogeneous cell population [12]. Heterogeneity can be introduced in these models by considering different compartments of cells, for example, according to their distinct proliferative ability [13, 14]. Cell transitions between different compartments are regulated

by probabilities. These models are mathematically simple to compute and their parameters can be inferred empirically by experimental data, but they do not include direct knowledge of single-cell kinetics. Population balance models deterministically simulate the temporal evolution of a continuous distribution of cell properties in the population [15]. Cellular processes are included by introducing experimental knowledge of the single-cell kinetics. The discrete counterpart of population balance models that accounts for the stochastic nature of some cellular processes is represented by Monte Carlo-based algorithms [16, 17]. These models describe heterogeneity as the cell-cell variability resulting from noise owing to the less number of molecules involved in some cellular mechanisms [18]. When a population composed by a small number of cells is to be simulated, cell-ensemble models offer a promising approach [19, 20]. They describe the behaviour of a single cell by a system of ordinary differential equations, and simulate an ensemble of cells to reproduce the distribution of properties in the population.

In this work, a mathematical model is developed in the form of a bivariate population balance equation (PBE) [15], coupled with an equation for the overall ligand consumption in culture. It describes a single receptor–ligand pair interaction. The deterministic nature of the model developed here is justified by the high number of surface receptors per cell [21], which does not require a stochastic approach in this case and makes reasonable the use of a continuous distribution for describing receptor heterogenity in the cell population. Also, the high number of cells considered discourages a cell-ensemble model, as in this case a population balance is computationally less intensive. At the single-cell level our model accounts for the following processes: the reversible reaction between receptors and ligand molecules to form receptor-ligand complexes, the production and degradation of receptors, the degradation of complexes and cell division. The partitioning of receptors and complexes from a mother cell to the two daughters suggests that cell division plays a crucial role in producing intrinsic heterogeneity. Thus, the model is implemented to track the evolution of the different generations present in culture by a system of population balances applied one to each generation. Simulations are performed, under the assumption of perfect mixing, in two conditions: first, at constant ligand concentration; then, when an equal amount of ligand is added all at once at the beginning of the cell culture. The second condition is representative of the situation in most of the conventional culture protocols. An analysis in terms of dimensionless variables is then performed to draw some general conclusions on the mechanisms that generate intrinsic heterogeneity in culture, and on the sensitivity of these results from the particular parameter set used.

As a case study, the model is applied to hematopoietic stem cells (HSCs). The therapeutic importance of HSCs resides in their ability to differentiate into all the different cell types of the hematopoietic system [22]. The single receptor–ligand pair considered in this example is c-Kit and stem cell factor (SCF) [23], selected on the basis of the crucial role it covers in hematopoiesis. In fact, SCF is added to culture medium in most of the expansion protocols [24]. The results found are not only restricted to this case study, and the range of their applicability is also discussed.

2 Theoretical framework

The intrinsic heterogeneity of a cell population is displayed in cell–cell variability in different subcellular compartments. In this work, we consider only heterogeneity in the number of cell surface receptors, and receptor-ligand complexes produced in the presence of ligand in culture medium. This assumption is motivated by the importance of receptor–ligand interactions in cell response to the environment [9]. A bottom-up multiscale approach is used considering the following processes:

- single-cell signalling related to production and consumption of receptors and complexes;
- ligand-dependent cell proliferation at the population level;
- overall consumption of ligand in the cell culture system.

Fig. 1a shows a graphical representation of the processes considered at the single-cell level. Receptors are produced by a cell at constant rate, and internalised and degraded at a rate proportional to their number on cell surface. Ligand is present in the medium as a dimer and reacts reversibly with two receptor molecules forming one complex molecule [25]. Receptor–ligand complexes are internalised at a rate proportional to their number on cell surface. The formation of complex molecules triggers a cascade of intracellular signalling that induces the cellular response. In the case of the pair c-Kit and SCF, cell survival and proliferation are enhanced [23]. In this work, we have considered only the effect on the cell proliferation rate (Fig. 1b). Cell division has an important impact in producing heterogeneity in the cell population. First, receptors and complexes are split between the two daughters when a mother cell divides, and thus the two cells formed are different from the mother that have produced them (Fig. 1b). Furthermore, cells divide at different rates because of the different number of complexes they have, consequently different generations of cells are present in culture at the same time (Fig. 1c). The overall mass balance of ligand in the cell culture system is performed, taking into consideration that ligand is consumed by each cell in the population at different rates, according to the number of receptors on its surface. Cell–cell interaction is not accounted for explicitly, but each cell is influenced by the other cells present in culture because of ligand consumption.

The combination of these phenomena leads to a population that shows cell-to-cell variability in the number of receptors and complexes. The mathematical model developed can be used to understand the relative importance of different contributions that lead to this intrinsic cell population heterogeneity. The model is analysed for two different experimental situations:

1. cell culture performed at constant ligand concentration;
2. cell culture performed in batch mode, for given amount of initial ligand.

These two cases represent, respectively, the ideal case in presence of a perfect control system that add exactly the ligand amount that is consumed by the cells, and the more common actual methodology used in biological experiments of HSC culture.

3 Mathematical model

3.1 General population balance model

A PBE [15] is a conservation equation that accounts for various processes, such as cell division and growth, treating cells of a specific state in terms of formation and depletion
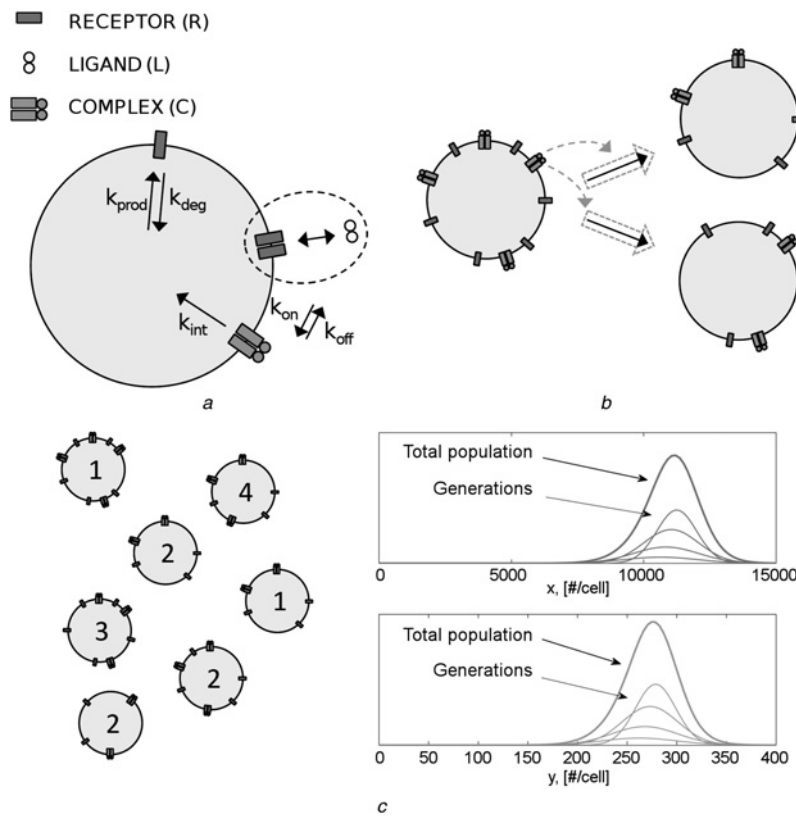
Fig. 1 *Schematic representation of the mechanisms taken into account in the mathematical model*

a At the single-cell level, receptors are produced and degraded; ligand is present as a dimer in the extra-cellular space and reacts with two receptor molecules to form a complex; the complex is degraded
b At cell division, receptors and complexes are split equally between the two daughter cells; the complex enhances the proliferation rate
c Population is composed by cells belonging to different generations (exemplified by the numbers inside the cells)
Model simulates the distribution of the number of receptors per cell and of the number of complexes per cell for the total population (thick line) and for each cell generation (thin line)

functions. The equation describes how the distribution of some properties, in our study the number of surface receptors and receptor–ligand complexes per cell, changes with time in the population. The complete formulation of the mathematical model developed in this work is given by the bivariate PBE

$$\frac{\partial W(x, y, t)}{\partial t} + \frac{\partial}{\partial x}[r_x(x, y) \, W(x, y, t)] + \frac{\partial}{\partial y}[r_y(x, y) \, W(x, y, t)]$$

$$= -\mu(y) \, W(x, y, t) + 2 \int_y^\infty \int_x^\infty \mu(y') \, p(x, y \mid x', y')$$

$$\times \, W(x', y', t) \, dx' dy' \quad (1)$$

where the independent variables are: x, number of receptors per cell; y, number of receptor-ligand complexes per cell and t, time. x and y are approximated as continuous variables. $W(x, y, t)$ is the joint probability density function in x and y multiplied by the cell density, N_{cell}; in particular, $W(x, y, t) \, dxdy$ represents the number of cells per unit culture volume having number of receptors between x and $x + dx$, and number of complexes between y and $y + dy$, at time t. Thus, the following normalisation condition holds for $W(x, y, t)$

$$N_{cell}(t) = \iint W(x, y, t) \, dxdy \quad (2)$$

$r_x(x, y)$ is the rate of variation of receptor number at the single-cell level, whereas $r_y(x, y)$ that of the number of complexes. $\mu(y)$ is the cell division rate, which, in the case of a perfectly homogeneous population (same number of complexes in all the cells), is equivalent to the commonly used specific growth rate, $\langle \mu \rangle$

$$\langle \mu \rangle = \frac{1}{N_{cell}} \frac{dN_{cell}}{dt} \quad (3)$$

$p(x, y \mid x', y') \, dxdy$ is the partitioning function, which represents the probability that a mother cell with status $[x', x' + dx']$ and $[y', y' + dy']$ will produce, upon division, a daughter cell with status $[x, x + dx]$ and $[y, y + dy]$. Thus, the following normalisation condition holds

$$\iint p(x, y \mid x', y') \, dxdy = 1 \quad (4)$$

Because of the conservation of the number of receptors and complexes at cell division, it must be satisfied the equality $p(x, y \mid x', y') = p(x' - x, y' - y \mid x', y')$. The left-hand side of (1) contains the accumulation and the terms related to the continuous change of receptor and receptor–ligand complex numbers because of the chemical reaction. By contrast, on the right-hand side, the first term accounts for mother cells disappearing after cell division, while the second term for the two daughter cells introduced by the same process.

Equation (1) is coupled with the equation for overall ligand consumption in culture, given by

$$\frac{dN_{L_2}}{dt} = -k_{on} \frac{N_x}{2} N_{L_2} + k_{off} N_y \tag{5}$$

where N_{L_2} is the number of ligand dimer molecules per unit volume of culture; k_{on} and k_{off} are kinetic constants; N_x and N_y are the total number of receptors and complexes, respectively, per unit volume of culture, derived as first moments of $W(x, y, t)$

$$N_x = \iint W(x, y, t) \, x \, dx dy \tag{6}$$

An analogous expression holds for N_y.

3.2 Single-cell process kinetics

The mathematical expressions of r_x and r_y depend on the rates of different phenomena: reversible receptor–ligand reaction

$$2\,R + L_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} (RL)_2 \tag{7}$$
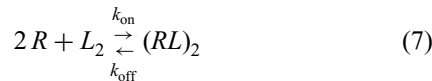
receptor synthesis and degradation

$$\overset{k_{prod}}{\rightarrow} R \overset{k_{deg}}{\rightarrow} \tag{8}$$

and complex internalisation

$$(RL)_2 \overset{k_{int}}{\rightarrow} (RL)_2^{int} \tag{9}$$

The meaning of the symbols is as following: R is the unbound receptor, $(RL)_2$ the surface receptor–ligand complex, L_2 the ligand dimer in the culture medium and $(RL)_2^{int}$ the internalised receptor–ligand complex (not included in the model). The final balances are given by

$$r_x = \frac{dx}{dt} = -k_{on} x N_{L_2} + 2 k_{off} y + k_{prod} - k_{deg} x \tag{10}$$

and

$$r_y = \frac{dy}{dt} = k_{on} \frac{x}{2} N_{L_2} - (k_{off} + k_{int}) y \tag{11}$$

The cell division rate, μ, is composed of a basal rate in absence of ligand, and a second term proportional to the number of complex molecules present in the cell as in [26]

$$\mu = k_{div} + k_{div_2} y \tag{12}$$

Receptors and complexes are assumed to be split equally between the two daughter cells, even though it is known that asymmetric cell division plays a role in the dynamics of HSC culture system [27]. Since asymmetric cell division would increase cell population heterogeneity, neglecting it will reveal the best case for the propagation of heterogeneity in culture. Thus, the partitioning function, p,

is defined as

$$p = \delta\left(x - \frac{x'}{2}, y - \frac{y'}{2}\right) \tag{13}$$

3.3 PBE model by generations

As previously pointed out, an important source of heterogeneity in the cell population is introduced at cell division. Thus, a system of PBEs was used, instead of (1), which tracks the temporal evolution of cells belonging to different generations

$$\begin{cases} \dfrac{\partial W^{(1)}}{\partial t} + \dfrac{\partial}{\partial x}[r_x W^{(1)}] + \dfrac{\partial}{\partial y}[r_y W^{(1)}] = -\mu W^{(1)} \\[2ex] \dfrac{\partial W^{(q)}}{\partial t} + \dfrac{\partial}{\partial x}[r_x W^{(q)}] + \dfrac{\partial}{\partial y}[r_y W^{(q)}] = -\mu W^{(q)} \\[2ex] \quad + 2 \int_y^\infty \int_x^\infty \mu\, p\, W^{(q-1)} \, dx' dy' \end{cases} \tag{14}$$

where the superscript $q = 2, \ldots, Q$ is the number of the generation, and Q is the total number of generations present during the culture period. The mother cells disappearing from $(q-1)$-generation are introduced in the q-generation PBE as daughter cells. The equations for each generation are similar except for the first generation, the oldest present in culture, whose equation does not show the daughter-entrance term. Equations (14) are coupled with (5) for ligand consumption. Cells in each generation, if they have not divided before, tend to a steady-state, dependent on ligand concentration, that is calculated by solving the coupled (10) and (11), given by

$$x_{ss} = \frac{(k_{int} + k_{off}) \cdot k_{prod}}{(k_{int} + k_{off}) \cdot k_{deg} + k_{int} \cdot k_{on} \cdot N_{L_2}} \tag{15}$$

and

$$y_{ss} = \frac{k_{on} \cdot k_{prod} \cdot N_{L_2}/2}{(k_{int} + k_{off}) \cdot k_{deg} + k_{int} \cdot k_{on} \cdot N_{L_2}} \tag{16}$$

where the subscript ss denotes steady state.

3.4 Simulation and analysis

Unless stated otherwise, the model parameters used in the simulations have the values shown in Table 1. They were obtained from data in the literature for the system c-Kit and SCF, when available. The kinetic constant of the direct receptor–ligand reaction, k_{on}, is derived from [28]. The kinetic constant of the reverse receptor–ligand reaction, k_{off}, is calculated by $k_{off} = k_{on} K_{eq}$, where K_{eq} is the

Table 1 Model parameter values

Parameter	Value	Unit	Reference
k_{on}	2.8×10^{-16}	ml/molecule/s	[28]
k_{off}	3.36×10^{-4}	1/s	[29]
k_{int}	1.5×10^{-3}	1/s	[28]
k_{prod}	2	molecule/cell/s	[26]
k_{deg}	10^{-4}	1/s	[26]
k_{div}	3.7×10^{-6}	1/s	[30]
k_{div_2}	7×10^{-9}	cell/molecule/s	[32]

dissociation constant for the reaction, having a value of 2 nM [29]. The rate constant for the internalisation of complexes, k_{int}, is reported in [28]. C-Kit production rate constant, k_{prod}, was not available for the system under consideration, and its value was fixed similar to the value of epidermal growth factor receptor (EGFR) synthesis rate from [26], as c-Kit and EGFR belong to the same family of tyrosine kinase receptors. The same assumption was used for the internalisation rate constant of free receptors, k_{deg}. The doubling time of hematopoietic cells cultured in the presence of 100 ng/ml of SCF, as described in [30], is $t_{d_1} = 26$ h. In the absence of SCF, the proliferation rate is highly decreased [31], and it was assumed a double doubling time in this case, $t_{d_2} = 52$ h, this value is consistent with the data reported in [32]. From these data, values of k_{div} and k_{div_2} were derived according to $k_{div} = \log(2)/t_{d_2}$, and $k_{div_2} = (\log(2)/t_{d_1} - k_{div})/y_{ss}$.

A numerical solution of the mathematical model outlined [(5) and (14)] has been developed in this work. Briefly, each $W^{(q)}$ is approximated by a bivariate normal distribution with time-dependent parameters. The Method of Moments [15] is then applied to convert the model to a set of ordinary differential equations, as described in detail in the Supplementary Material. All model simulations in this work are performed using the ode113 solver in MATLAB (The MathWorks, Natick, MA).

A preliminary simulation is performed to generate a heterogeneous population as the initial condition for all the simulations shown in the Results section. At the beginning the population is considered containing all cells in the first generation, with mean number of receptors equal to x_{ss}, mean number of complexes equal to y_{ss} and null standard deviation of both receptors and complexes. This first simulation is run for a time interval of 3 days in the absence of ligand. The final distributions of receptors and complexes are used as input for the subsequent simulations, where initial cell density is fixed at 10^6 cell/ml.

The proposed model can simulate the temporal evolution of the distributions of receptors and complexes per cell for each generation. Simulations are performed for the two cell culture conditions of constant ligand concentration, and with ligand added all at once at $t = 0$, thus progressively decreasing in concentration. The results will be summarised in the form of the following statistics: the mean number of receptors and complexes per cell, $\langle x \rangle$ and $\langle y \rangle$, both for the total population and for each cell generation; the coefficient of variation, CV_x and CV_y, of the number of receptors and complexes per cell, both for the total population and for each cell generation; the standard error of the mean for the generations with respect to the total population normalised by the mean, $SEM_x/\langle x \rangle$ and $SEM_y/\langle y \rangle$. In particular, CV_x is calculated by the expression

$$CV_x = \frac{\sigma_x}{\langle x \rangle} \tag{17}$$

where σ_x is the standard deviation of the number of receptors, either of the total population or of the single generations; an analogous expression holds for CV_y. Whereas SEM_x is given by

$$SEM_x = \left(\frac{\sum_{q=1}^{Q} (\langle x \rangle^{(q)} - \langle x \rangle)^2}{Q} \right)^{1/2} \tag{18}$$

where $\langle x \rangle^{(q)}$ is the mean number of receptors in the qth generation; an analogous expression holds for SEM_y.

4 Results

4.1 Simulations at constant ligand concentration

A simulation is computed initially at constant ligand concentration. As shown in Fig. 2a (dashed line), constant ligand concentration requires the continuous addition of ligand (solid line), because of single-cell ligand consumption coupled with the increase in cell density. The cell density in culture, N_{cell}, increases exponentially (Fig. 2b). On the contrary, each cell generation contributing to form the whole cell population, after its entrance, first increases in cell number, and then decreases and disappears. Thus, the cell population has a time-dependent composition in terms of fraction of cells belonging to different generations. When a new generation enters, its mean properties, $\langle x \rangle$ and $\langle y \rangle$, are different from those of the total population. In fact, cells in each generation appear with half the number of receptors and complexes of their mother cells, and then tend to the steady-state given by (15) and (16). Nonetheless, the contribution of a newborn generation to the whole is limited by the few cells it contains (Figs. 2c and e). Thus, the mean properties of the total population are more or less close to the steady state, depending on the proliferation rate.

The coefficients of variation for receptors and complexes, CV_x and CV_y, quantitatively describe the heterogeneity in the cell population and inside each generation. Unlike standard deviations, coefficients of variation of different distributions can be compared as they are normalised by the respective mean. With the exception of the first generation that has approximately null CV_x and CV_y, because all of its cells reach the steady state, all of the next generations show a certain level of heterogeneity because of cells from the previous generation continuously entering with a smaller number of receptors and complexes (Figs. 2d and f). In particular, three phases can be distinguished in CV_x and CV_y of single generations: first, a steep decrease because of the sudden increase in $\langle x \rangle$ and $\langle y \rangle$ [see (17)]; second, an increase in heterogeneity because of the increase in σ_x and σ_y; and, third, a slow decrease as the fraction of cells close to steady state becomes more and more dominant.

Although the properties of each cell generation are time dependent, the total population distribution of receptors and complexes exhibits a pseudo steady state. In Fig. 3 results are presented to show this steady state as a function of different constant ligand concentrations. A higher ligand concentration produces an increase in $\langle y \rangle$ (Fig. 3g) that affects the proliferation rate, according to (10), increasing the final cell density (Fig. 3b). As the dependence of $\langle y \rangle$ on ligand concentration is not linear this effect is more relevant at small concentration values. The total population heterogeneity, CV_x and CV_y (Figs. 3e and h), arises from three different sources:

1. the cell population is composed of a distribution of different generations (Fig. 3c);
2. it has an inter-generational heterogeneity, because the mean properties of each generation, $\langle x \rangle$ and $\langle y \rangle$, differ from each other (Figs. 3d and g);
3. it has an intra-generational heterogeneity, as each generation is heterogeneous on its own (Figs. 3e and h).
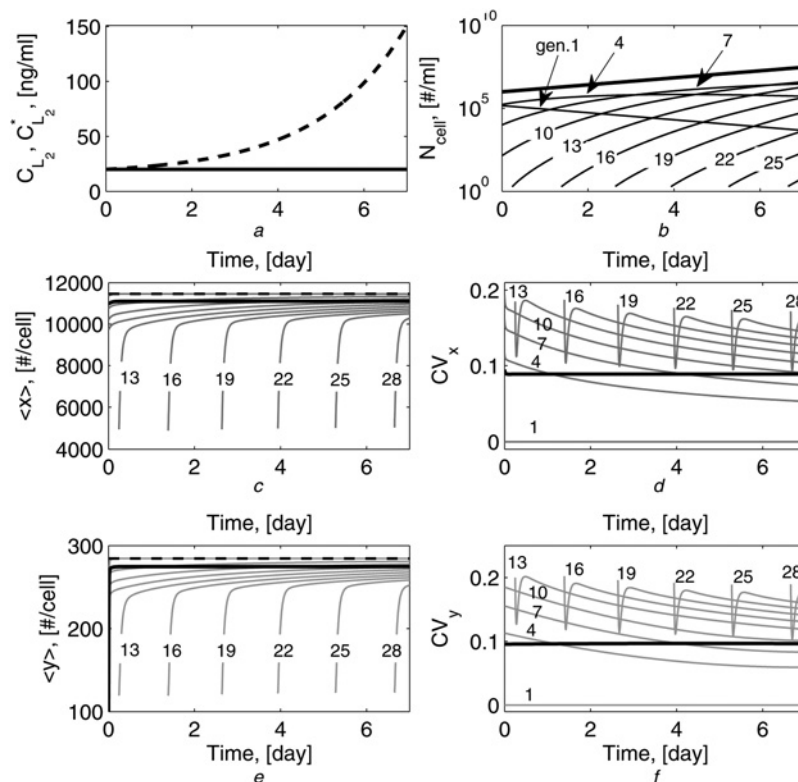
Fig. 2 *Results at constant ligand concentration* $C_{L_2} = 20$ *ng/ml*

a Constant ligand concentration, C_{L_2}, in culture (solid line), and total amount of ligand added, $C_{L_2}^*$, to keep concentration constant (dashed line), as functions of time

b Cell density, N_{cell}, of the total population (thick line), and of the generations indicated (thin lines) as a function of time

c Mean number of receptors per cell, $\langle x \rangle$, for the total population (black solid line), and for the generations indicated (gray lines), as a function of time. Single-cell steady state of the number of receptors per cell (black dashed line)

d Coefficient of variation of the number of receptors per cell, CV_x, for the total population (black line), and for the generations indicated (red lines).

e−f Analogous to *c−d* for the number of complexes per cell

The first source of heterogeneity is negligible as long as different generations have the same properties. The importance of the second and third sources is dependent on the particular kinetics considered for the single-cell processes and for cell division. While CV_x and CV_y are measures of the global heterogeneity of the population, $SEM_x/\langle x \rangle$ and $SEM_y/\langle y \rangle$ include only the inter-generational contribution. Thus, the relative weight of the second and third sources of heterogeneity is quantitatively determinable.

With the single-cell kinetics considered in this work, the total population heterogeneity, quantified by CV_x and CV_y, decreases at high ligand concentration because both inter- and intra-generational heterogeneities decrease. In fact, at the single-generation level, an increase in ligand concentration causes a reduction in the time cells need to reach the steady state, given by (15) and (16).

4.2 Simulations of different ligand administration profiles

In the previous case of constant ligand concentration, ligand needs to be continuously added to the system to compensate for ligand consumption from the cell. However, in the absence of a precise control, the ligand concentration in culture changes with time. In Figs. 4 and 5 a comparison is shown between the results of simulations at constant ligand concentration, already analysed, and the case where ligand is added to the cell culture all at once at $t = 0$. To

ensure a fair comparison, an equal amount of ligand is administered in the two cases for the whole period of 7 days.

When ligand is added at the beginning, its concentration decreases with time on account of cell uptake (Fig. 4a). On the overall cells consume more ligand in this case, in fact its concentration becomes practically null before the end of the 7-day period. The cell density is larger (Fig. 4b) because the division rate, influenced by complex formation in the presence of ligand, is initially higher, then decreases as soon as ligand is depleted in culture and no complexes are formed anymore. The mean number of receptors per cell, $\langle x \rangle$, increases as ligand concentration decreases because more receptors remain unbound (Fig. 4c). At null ligand concentration, $\langle x \rangle$ reaches a steady state that represents the dynamic equilibrium between receptor production and degradation. Conversely, $\langle y \rangle$ decreases at decreasing ligand concentrations until no complexes are formed anymore (Fig. 4e). Heterogeneity in receptor number, represented by CV_x, increases until a maximum value for null ligand concentration (Figs. 4d and f). Instead CV_y tends to infinity when ligand is depleted because $\langle y \rangle$ becomes null [see (17)].

Fig. 5 is analogous to Fig. 3 for the case of ligand added all at once at the beginning, results from simulations at constant ligand concentrations are also shown for comparison (dashed lines). Whatever amount of ligand is added, it is always depleted after a 7-day culture period (Fig. 5a) because of the higher cell density (Fig. 5b). While the proliferation rate is highly enhanced when ligand is added from the beginning (Fig. 5b), the cell population composition in terms of
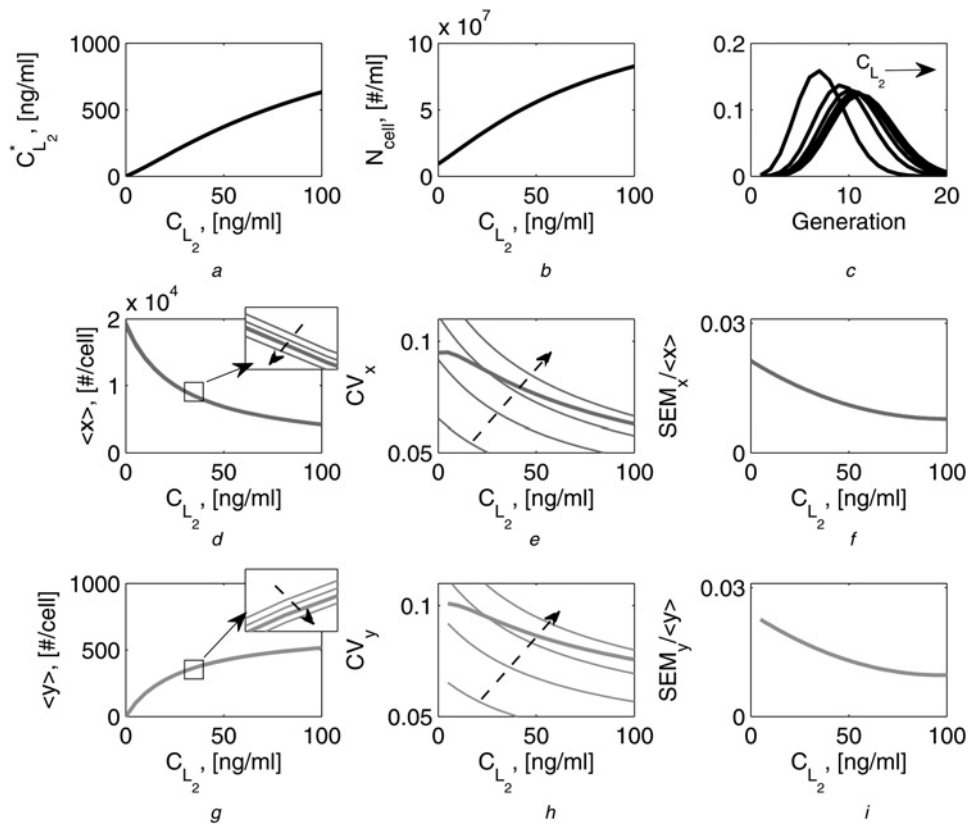
Fig. 3 *Results at 7 days for different constant ligand concentrations, C_{L_2}*

a Total amount of ligand added, $C_{L_2}^*$, to keep constant concentration in culture, C_{L_2}

b Cell density of the total population

c Distribution of generations in the population for $C_{L_2} = 0, 15, 30, 45, 60$ ng/ml

d Mean number of receptors per cell for the total population (thick line) and for generations 4, 7, 10, 13 (thin lines), in increasing order indicated by the dashed arrow

e Coefficient of variation of the number of receptors per cell for the total population (thick line) and for generations 4, 7, 10, 13 (thin lines), in increasing order indicated by the dashed arrow

f Normalised SEM of the mean receptor number per cell, considering only the generations that contribute to 98% of the total population cell number

g–i Analogous to *d–f* for the number of complexes per cell. In (*h–i*) curves do not start from $C_{L_2} = 0$ as their value approaches infinity at that point

fraction of cells in each generation shows little change (Fig. 5c). Thus, the increased proliferation rate is not related to a higher number of generations in culture, but to an increase in the proliferation rate of the first generations, as they experienced a very high ligand concentration in the first period of culture. The simulation results show that the final population properties after 7 days are almost invariant to changes of ligand total amount (Figs. 5d–i). In fact, for the system under consideration, a higher amount of ligand increases only the number of cells.

4.3 Dimensionless analysis of cell population heterogeneity

A dimensionless analysis is performed to understand the fundamental mechanisms that play a role in determining intrinsic heterogeneity in a cell population at constant ligand concentration. In addition to CV_x and CV_y, another dimensionless variable is considered, the ratio between the following two characteristic times: the characteristic time of cell division, τ_{div}, defined as the doubling time of cells having a number of complexes equal to y_{ss}

$$\tau_{\mathrm{div}} = \frac{\log(2)}{(k_{\mathrm{div}} + k_{\mathrm{div}_2} \cdot y_{\mathrm{ss}})} \tag{19}$$

where y_{ss} is calculated for different ligand concentrations by (16); and the characteristic time for a newborn cell to reach the steady state, τ_{ss}, calculated by solving the system of (10) and (11). Specifically, τ_{ss} is the time to reach $x = 99\% x_{\mathrm{ss}}$, under constant ligand concentration, from the initial state $(x, y) = (x_{\mathrm{ss}}/2, y_{\mathrm{ss}}/2)$.

Results shown in Fig. 6 are obtained for different sets of parameters; in particular, one parameter was doubled at a time (as indicated in figure legend). Fig. 6a shows the relationship between ligand concentration and the characteristic time ratio, $\tau_{\mathrm{div}}/\tau_{\mathrm{ss}}$, for the single-cell kinetics considered in this work, which is dependent on the parameter set under consideration. Figs. 6b and c show the dependence of population intrinsic heterogeneity in the number of receptors and complexes, CV_x and CV_y, from the ratio $\tau_{\mathrm{div}}/\tau_{\mathrm{ss}}$. CV_x and CV_y are calculated by solving (14) at different constant ligand concentrations. Intrinsic heterogeneity shows a dependence on the characteristic time ratio. In particular, a reduction in cell population heterogeneity is achieved when cells proliferate more slowly. As the different curves are overlapping, this dependence is insensitive to arbitrary parameter changes in the model (Figs. 6b and c). Thus, the ratio $\tau_{\mathrm{div}}/\tau_{\mathrm{ss}}$ is a dimensionless number that can be used to characterise intrinsic heterogeneity in the case presented in this work, calculated for ligand concentration kept constant in culture. If ligand concentration changes in culture with a characteristic
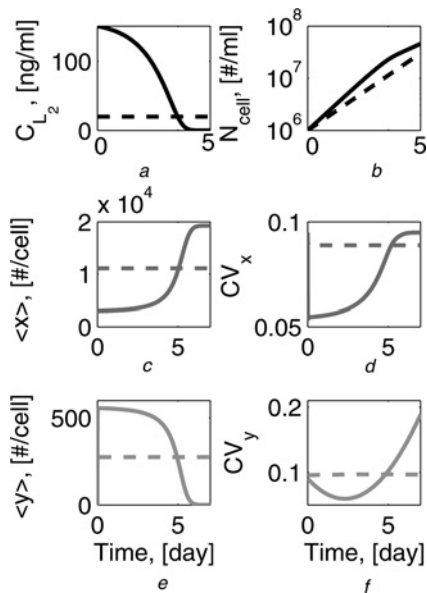
Fig. 4 *Comparison of results at the constant ligand concentration, C_{L_2}, of 20 ng/ml (dashed lines), and for a weekly equal amount of ligand added at $t = 0$ (solid lines)*

a Ligand concentration in culture
b Cell density of the total population
c Mean number of receptors per cell for the total population
d Coefficient of variation of the number of receptors per cell for the total population
e–f Analogous to *c–d* for the number of complexes per cell

time much larger than τ_{div} and τ_{ss}, then results in Figs. 6*a* and *b* are a good approximation, even in those conditions.

5 Discussion and conclusions

Expansion of stem cells in vitro is required for making their therapeutic use more effective [3]. Besides an increase in cell number, the control of the final cellular phenotype is necessary for a safe clinical application [4]. In particular, heterogeneity in the expanded cell population needs to be reduced and controlled, as small fractions of different cells can give a negative clinical response [5]. Cellular behaviour and fate are dependent on culture conditions [7]; thus, extrinsic heterogeneity in a cell population arises because of spatial gradients in metabolite and growth factor concentrations over the culture volume. Dynamic culture systems, like bioreactors, are convenient means to reduce these spatial inhomogeneities [8].

Even under perfect mixing conditions, a cell population presents a certain level of intrinsic heterogeneity. In this work, a theoretical study was performed to understand some aspects of intrinsic heterogeneity under perfectly homogeneous culture conditions. The heterogeneity considered was that in the number of receptors and receptor–ligand complexes per cell. The focus on receptor–ligand mechanism was motivated by the importance it has in cell interaction with the environment [9]. Furthermore, it connects an operative variable in cell culture, ligand concentration, to a measurable single-cell property, receptor number. In particular, the
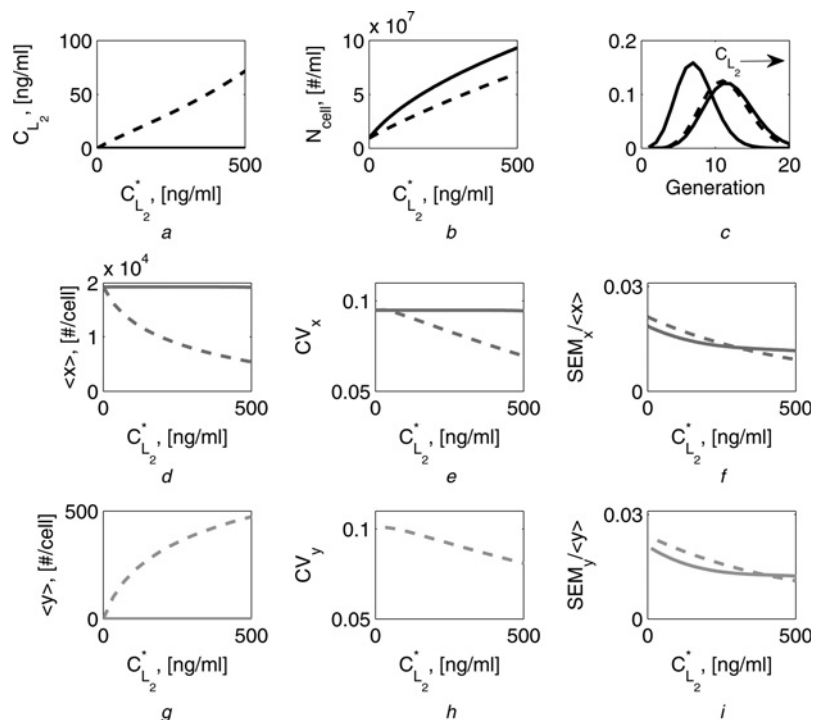

Fig. 5 *Results at 7 days as function of the total amount of ligand added in the two cases: constant ligand concentration (dashed lines) and ligand added at $t = 0$ (solid lines)*

a Ligand concentration in culture, C_{L_2}
b Cell density of the total population
c Distribution of generations in the population for $C_{L_2}^* = 0$ and 500 ng/ml. $C_{L_2}^*$ refers to the total amount of ligand added in 7 days
d Mean number of receptors per cell for the total population
e Coefficient of variation of the number of receptors per cell for the total population
f Normalised SEM of the mean receptor number per cell, considering only the generations that contribute to 98% of the total population cell number
g–i Analogous to *d–f* for the number of complexes per cell. In *h–i* curves do not start from $C_{L_2} = 0$ as their value approaches infinity at that point. In (*h*) the curve for the total population is not shown as it approaches infinity for all C_{L_2}
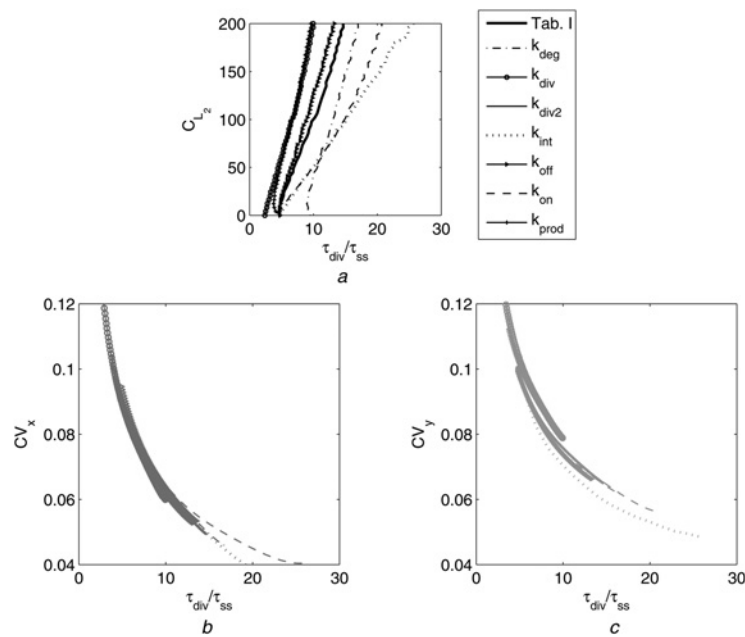
Fig. 6 *Dimensionless analysis of intrinsic heterogeneity at constant ligand concentration*

a Relationship between ligand concentration in culture and the ratio of the characteristic times of division, τ_{div}, to that spent to reach single-cell steady state, τ_{ss}
b Coefficient of variation of the number of receptors per cell for the total population as a function of the characteristic time ratio
c Analogous to *b* for the number of complexes per cell
Different types of lines refer to results obtained by doubling the parameter indicated in the legend

developed mathematical model focuses on c-Kit SCF pair in HSC culture. Considering only one receptor type for characterising stem cell population heterogeneity limits the biological plausibility of the model, but gives insight on general mechanisms that can be easily extended to more complex culture systems descriptions.

First, intrinsic heterogeneity was studied in the case of constant ligand concentration in culture, which represents the ideal condition to have a constant quality of cellular outcome. Even in this situation, intrinsic heterogeneity is generated at cell division, because of the partitioning of receptors and complexes from a mother cell to the two daughters. The overall population presents steady distributions of receptors and complexes, whereas the different generations of cells that it contains have time-dependent properties. Working at different ligand concentrations affects the cellular outcome. The effect of a change in ligand concentration was more pronounced at low concentrations because of the non-linear dependence of the different processes on ligand concentration. In particular, intrinsic heterogeneity diminishes at higher ligand concentrations for the system under consideration. The contributions of both intra-generational and inter-generational heterogeneity to total population heterogeneity were highlighted and quantified by two statistics: the coefficient of variation and the normalised standard error of the mean. This aspect is relevant considering that experimental techniques are available to select cells from different generations [30], which can potentially avoid inter-generational heterogeneity. Specifically, inter-generational heterogeneity plays a major role in the system we simulated.

To study the importance of ligand administration kinetics, the effect of ligand added all at once at the beginning of culture was simulated. Cell population properties become time dependent in this situation also for the total population, and thus the time duration of cell culture becomes an additional parameter to consider. A comparison

was performed between this case and that of constant ligand concentration. The two cases present a different final cellular heterogeneity. When ligand is added at the beginning, it is consumed more rapidly, and cell population heterogeneity increases very fast as soon as ligand concentration falls under a certain threshold. On the overall, it seems important not to completely deplete ligand concentration in culture to maintain reasonable values of heterogeneity. The effect of different amounts of ligand added at culture start, simulating the same span of time of 7 days, was analysed. Cell population properties were unaffected because, because of an increase in proliferation rate at higher ligand concentrations, ligand was always depleted after 7 days.

A dimensionless analysis was performed to draw some general conclusions on the sources of intrinsic heterogeneity at constant ligand concentration. The key parameter was found to be the ratio between the characteristic time of cell division and that a newborn cell needs to reach the single-cell steady-state. This ratio depends on ligand concentration, model parameters and the particular single-cell kinetics considered. The dependence of intrinsic heterogeneity, represented by CV_x and CV_y, on this ratio is reduced at higher values of τ_{div}/τ_{ss}, that is, when cell proliferation rate is lower. This behaviour was found to be independent of model parameters. Furthermore, Figs. 6*b* and *c* have a much wider applicability than the model presented here. In addition to being invariant to the model parameters used, it accounts also for different mathematical forms of single-cell kinetics, as long as cells tend to one stable steady state and do not interact with each other. Other simple expressions of the division rate, μ, can give similar results to those shown in Figs. 6*b* and *c*, but a deeper analysis is required in this case. The dimensionless analysis was performed at constant ligand concentration, and the results approximately describe also the case where ligand concentration changes on a timescale larger than τ_{div}

and τ_{ss}. It is not straightforward to obtain such general results for the case of fast-changing ligand concentration, and future work will address this aspect.

The division rate was modelled as in (12). At very low ligand concentrations, when the number of receptor–ligand complexes is low (less than about 50) the variable part $k_{div_2}y$ of the rate expression becomes negligible, and all the cells in the population end up having the same probability of division, both the newborn and the older ones. This is unrealistic because cell cycle requires a minimum duration to complete essential biological processes. The rate expression could be improved by including a time delay before division accounting for this. The general results shown in Fig. 6 would be only qualitatively affected, as the time delay increases τ_{div} but has no impact on τ_{ss}, producing a shift to the right of the curves in b and c.

The overall intrinsic heterogeneity was unavoidable because it is a consequence of basic cellular processes. Nonetheless, its reduction can be accomplished by choosing proper culture conditions, such as, for the system considered in this work, high ligand concentrations or at least avoiding the complete ligand depletion. The main result obtained is the correlation between heterogeneity and cell division rate, relatively to the other cellular processes. Thus finding proper culture conditions for cell number maximisation and population heterogeneity minimisation involves the solution of an optimisation problem.

6 Acknowledgments

7 References

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