Mesenchymal Stem Cell Heterogeneity and Ageing In Vitro: A Model Approach

Jörg Galle, Martin Hoffmann and Axel Krinner

Abstract Mesenchymal Stem Cell (MSC)-based therapies have been suggested as a particular promising strategy in tissue regeneration. These cells can easily be obtained from the patient and are able to produce a large number of progeny that can be induced to form connective tissue. However, rapid amplification of the isolated cells is required for their therapeutic application. While already the isolated populations are heterogeneous regarding various functional and molecular aspects, this heterogeneity further evolves during amplification. Understanding the origin and development of MSC heterogeneity will help to improve MSC culture conditions and thus facilitate their clinical use. We here review recent results on MSC heterogeneity and introduce a mathematical framework that approaches MSC heterogeneity on the single cell level. This approach bases on the concept of noise-driven MSC differentiation and allows describing MSC heterogeneity with respect to their differentiation state and age. It is capable of describing the impact of MSC heterogeneity on in vitro expansion and differentiation. We present new results on the formation of an age structure in MSC populations in vitro and the age-dependent differentiation structure of MSC populations. Moreover, we discuss open questions regarding MSC adaptation to changing environments and the cell intrinsic control of state fluctuations.

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1 Introduction

Mesenchymal stem cells (MSCs, also known as multipotent mesenchymal stromal cells) have been characterised as a heterogeneous cell population of adherent spindle-shaped cells capable of differentiating into bone-marrow stromal cells, osteoblasts, chondrocytes, adipocytes and myocytes. Related lineage priming of MSCs has been nicely demonstrated in monoclonal culture [23]. In some tissue types, e.g. bone-marrow stroma, adipose, skeletal muscle and synovium, MSCs persist in adult life without loosing their capacity to proliferate and differentiate [41, 76]. Accordingly, MSCs have been proposed as innovative therapeutic tools in tissue regeneration. Their therapeutic deployment comprises treatment of various diseases, including osteoarthritis [12] and myocardial infarction [40]. MSC application in asthma, radiation exposure, and neurological disorders has been explored as well [9]. Moreover, beside of having high regenerative potential these cells have been shown to carry immunosuppressive capacities, to improve angiogenesis and to prevent fibrosis [24].

Therapeutic applications of MSCs require massive in vitro expansion of the isolated cells [7, 21]. The populations typically can be expanded for up to 20 population doublings (PDs) until they enter a senescent state. Figure 1a shows a single cell-derived, expanding clone of MSCs. As shown in Fig. 1b the growth properties of such clones can vary largely during expansion. Various culture protocols have been suggested in order to isolate MSCs with high regenerative potential [18, 88, 92]. However, independent of the culture condition applied, massive replication of MSCs was found to be associated with continuous decline of the cell's functional competence, which was called "MSC ageing" [91]. During expansion the MSCs show a decreasing proliferation potential [84, 88]. Moreover, the efficiency of differentiating into local tissue after transplantation was found to severely decrease during expansion [75]. During prolonged in vitro culture MSCs frequently undergo spontaneous malignant transformation which represents a biohazard in long-term expansion [77]. Both ageing and transformation appear to be stochastic in nature and render the MSC populations an additional layer of heterogeneity.

In the following we discuss different hypotheses on the origin and development of MSC heterogeneity and introduce a model framework which allows addressing related questions. We show that a noise-driven approach to MSC differentiation combined with a model of intrinsic MSC ageing can explain MSC heterogeneity on both the individual and population level. We highlight open questions on MSC heterogeneity and suggest future investigations.

2 MSC Heterogeneity on Different Scales

Due to a growing body of evidence it is has been generally accepted that tissue stem cells are heterogeneous with regard to their function. This heterogeneity has been suggested to involve properties like their cycling activity, engraftment

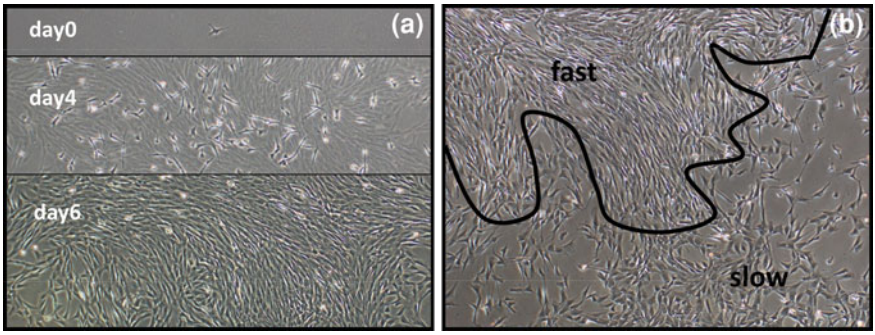

Fig. 1 Expansion of MSCs in vitro. **a** Massive expansion of an ovine MSC clone within 6 days of in vitro culture at standard culture conditions [101]. **b** Spatial competition of ovine MSC clones in vitro. A fast growing clone spreads into the area already occupied by a slow growing clone

potential or differentiation status as well as their expression of adhesion molecules or cell surface antigens [54]. Accordingly, a functional definition of tissue stem cells has been given by Loeffler and Roeder [53] that includes heterogeneity as a characteristic feature: 'Tissue stem cells are a potentially heterogeneous population of undifferentiated cells that are capable of proliferation and production of a large number of differentiated tissue cells for replenishing tissue continuously and after injury, while self-maintaining their population.'

However, isolation of stem cells for subsequent applications by applying these functional criteria is impossible, simply because a cell cannot be differentiated in different lineages while simultaneously maintaining its 'stem cell' state. As an alternative method marker systems have been studied in order to identify individual cells that carry stem cell properties. But, while well defined marker systems are available for other stem cells, e.g. for haematopoietic stem cells (HSCs, [27]), for MSCs such marker systems are still a matter of debate [42]. First effective protocols have been suggested more than ten years ago [67]. Currently, standard isolation protocols for human MSCs use combinations of up to ten surface markers. There is common agreement that more than 95% of the cells must express CD73, CD90 and CD105 and less than 2% CD35, CD45 together with CD11b or CD14 [24, 25, 48]. However, the MSCs isolated applying related protocols show still a large variance in the expression of these markers. Subpopulations of them with more defined expression have been demonstrated to carry different functional potential (e.g. Sca-1, [13]). Moreover, gene expression analysis of individual MSCs [82, 83] demonstrated MSC heterogeneity even on the single cell level. Despite a common molecular signature of potential multilineage differentiation capacity the analysed cells show considerable variance in expression.

During expansion MSCs adapt to the in vitro conditions and change their expression profile until they reach a senescent state as described in detail by Wagner et al. [94]. How this adaptation process affects MSC heterogeneity will be discussed below. The related changes in the expression profile are accompanied by morphological changes of the cells. Initially many of the cells are round and

small, so-called RS cells, [14, 15]. But relatively fast most of the cells develop a fibroblast-like morphology. With increasing culture time they spread more and more and their morphology becomes irregular. This suggests using morphological cell properties, as e.g. cell size, as alternative sorting targets. Actually, characterisation of high proliferative subpopulations by cell size has been demonstrated [31]. Unfortunately, cell size does also change according to spontaneous transformation events during long term culture. Such transformations can occur frequently and can start early. They result in the emergence of a cell type characterised by an elevated proliferation, reduced plasticity and round and small morphology [2, 77]. Thus, transformed cells may be hardly distinguishable from high potential untransformed MSCs by morphological characterization only.

Interestingly, the time point of spontaneous transformation seems to be stochastic in nature like that of acquiring senescence [77]. Experimental results suggest that the transformed fate is not correlated to a defined regulatory state. So, transformed clones have been observed to differ in their expansion rate [77] as well as in the number of chromosomes present in the cells [2]. In the following we will focus on non-transformed MSCs and will neglect transformation as a particular source of population heterogeneity.

In summary, experimental findings demonstrate that MSC heterogeneity is a multi-scale phenomenon and is subject to significant changes during in vitro cultivation.

3 On the Origin of MSC Heterogeneity

While heterogeneity is accepted to be a characteristic property of stem cell populations, its origin is still not well understood. Heterogeneity of non-transformed functional stem cells has been discussed as a consequence (i) of cell adaptation to dynamic environments and (ii) of the flexibility and reversibility of stem cell fate decisions. Additional variance in cell fates may be associated with cell ageing.

3.1 Heterogeneity as a Consequence of MSC Environmental Adaptation (Extrinsic)

It has been demonstrated that MSCs from different tissues, including bone-marrow stroma, adipose, skeletal muscle, synovium and umbilical cord differ in both molecular and functional properties [39, 58, 63, 99]. Typically a large number of genes and proteins have been found to be differentially expressed (see e.g. [61, 62]) and, although the MSCs were expanded over many PDs in vitro, these differences in expression appeared to be conserved. Moreover, they also manifest in functional differences of the MSCs including their expansion and differentiation potential. For example experiments on human bone marrow MSCs revealed that about one-third of the clones are able to acquire phenotypes of pre-adipocytes,

osteocytes and chondrocytes [67]. In contrast, only 1.4% of single MSCs isolated from adipose-derived adult stem cell populations were tri-potent, the others being bi-potent or uni-potent [102]. Dental MSCs were found to have less potential to differentiate into adipogenic and chondrogenic, but more potential for neural and odontogenic differentiation compared to bone marrow MSCs [39].

In agreement with these in vivo results in vitro culture conditions strongly impact MSC phenotypes. A large number of environmental factors such as oxygen [19, 41, 52, 101], glucose [91] and growth factors [5, 36] have been demonstrated to affect MSC expansion and differentiation. These processes are also affected by substrate stiffness [29], geometry [66], micro/nano-structure [20, 60] as well as surface chemistry [17]. Thus, one may expect that population heterogeneity observed in a specific environment (compare Fig. 2) is a consequence of adaptation of the MSCs to different microenvironments. Thereby, stem cells may either reside in microenvironments that support stem cell maintenance, so-called stem cell niches, or in activating environments that enforce their expansion. While e.g. HSC-niches are well described [27], MSC-niches are discussed controversial. Several studies suggest that MSCs reside in a perivascular niche in almost all adult tissues, where they associate with blood vessels [16]. However, they are also found in non-vascularised cartilage tissue where they show a well defined spatial distribution [68]. In fact, until now it has not been shown directly that heterogeneous microenvironments are responsible for the experimentally observed MSC population heterogeneity.

3.2 Heterogeneity as a Consequence of MSC Self-Organisation (Flexibility)

MSC differentiation and lineage specification have been found to be at least partially reversible, demonstrating a limited but significant plasticity of MSCs [87]. Moreover, plasticity has been described as a fundamental feature of these cells [100]. This is supported by recent experimental findings that they represent an excellent source for generating induced pluripotent stem cells [98] which appeared to be the closest equivalent to embryonic stem cells as demonstrated by DNA microarray gene profile and germline-transmission efficiency [64]. Thus, the question raises whether individual MSCs exhibit a stable tissue specific phenotype or whether they permanently change their regulatory states exhibiting a tissue specific phenotype only on population level.

Evidence that fate decisions of stem cells are reversible and that these cell populations self-organise permanently emerges from so-called regeneration experiments [10, 11]. In particular it has been shown that for a haematopoietic progenitor cell line the expression profile of the stem cell marker Sca-1 regenerates from different subpopulations. These experiments suggest fluctuations in the expression of such markers for each individual cell in a fixed environment. For primary MSCs such a regeneration of the tissue specific distribution of regulatory

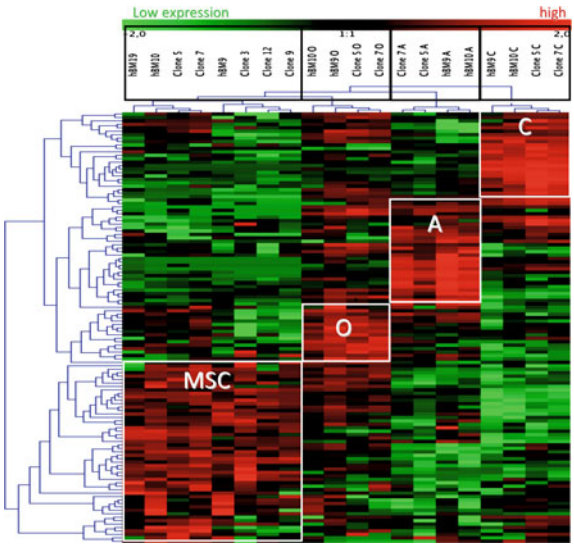

Fig. 2 Clonal heterogeneity of bone marrow MSCs. Hierarchical clustering using individual samples and genes down regulated after adipogenic (A), osteogenic (O), and chondrogenic (C) differentiation and genes implicated in A, O, and C differentiation. Individual samples are three primary MSC layers and five MSC clones before differentiation and two primary MSC layers and two MSC clones after A, O, and C differentiation. The individual samples show considerable differences in expression before and after differentiation. From: Delorme et al. [23]

states is expected within a few days [46]. However, such experiments have not been carried out so far. While such observations do not rule out the impact of microenvironments on the formation of heterogeneous populations, they suggest that stem cells populations can develop heterogeneity also independent of the presence of complex in vivo environments. However, a direct proof of the assumption of (stem cell) state fluctuations would require demonstrating them in individual cells in a defined environment. A prerequisite of such studies is long term single cell tracking, which has been successfully demonstrated for HSCs [72], and for which excellent new technology has been established [43].

In summary, there is evidence that MSCs are very flexible in their fate decisions and can adapt to a large variety of different environments. The experimental findings can be understood assuming that individual cells underlie permanent fluctuations of their regulatory states and that these fluctuations are modulated by the cell environment.

3.3 Heterogeneity as Consequence of MSC Ageing (Intrinsic)

The functional potential of MSCs has been shown to decrease throughout life. Under homeostatic conditions, there are limited demands on the self-renewing stem cells and so these cells divide infrequently, sparing stem cells the perils of

DNA-replication and mitosis. However, under stress the metabolic activity of the stem cells increases. The stem cells are exposed to higher levels of DNA-damage-inducing metabolic side products such as reactive oxygen species [85]. It has been suggested that the damages induced thereby impact not only the stem cells but the whole organism. In fact, excess replicative demands alone can induce progeroid phenotypes [78].

Recent experiments demonstrated that artificial in vitro ageing and in vivo ageing of MSCs induce related changes on the cellular as well as on the molecular scale [45, 94]. Thereby, pre-mature senescence has been implicated as a major cause of the in vitro decline in MSC function [90]. The accumulation of this phenotype, also called replicative senescence, has been demonstrated to be a continuous process in MSCs [95]. Interestingly, expansion at low oxygen pressure and low glucose culture decreases the number of accumulating senescent cells compared to high oxygen pressure and high glucose culture, respectively [89, 101]. Whether the accumulation of damage is actually pre-requisite for MSC ageing is still unknown. However, DNA-damage rarely affects the 2% of protein-coding sequences. Instead, it is expected to alter regulatory regions [69] and expression and function of non-coding RNAs that are involved in chromatin regulation [4]. Accordingly, an increasing number of ageing studies investigate the impact of epigenetic changes, such as DNA hyper/hypo-methylation, and histone modifications [65, 86]. The results suggest that decreasing accessibility of certain regulatory states of MSCs due to epigenetic remodelling may represent an alternative or at least a complementary explanation of ageing.

Without any question, the distribution of MSC states changes over time, i.e. with age and in a tissue specific manner. Whether this is due to changing environments or due to epigenetic remodelling and accumulation of damage is currently not fully understood.

4 General Model Approaches

A large number of theoretical approaches to MSC culture aim at quantitatively describing culture conditions and their impact on processes such as matrix formation [81]. Theoretical models of MSC expansion and differentiation are rather rare [22, 51] and do not include single cell-level population heterogeneity. In order to provide reliable predictions on the dynamics of such systems, theoretical approaches are required that account for: (i) composition and structure of the cell environment and (ii) particular stem cell properties such as functional differentiation and self-renewal in individual cells. Currently there are different concepts to approach these problems in general. In particular there are different concepts of modelling stem cell organisation. The most prominent are the ‘pedigree concept’ and the ‘plasticity concept’.

The pedigree concept treats ‘stemness’ as a property that if once lost is lost forever. In the models that obey this concept this loss can be a deterministic or a stochastic process. Accordingly the development of individual progenitors may more or less differ. However, over time they all will approach a defined

state (Fig. 3a). Stem cells inherit 'stemness' by performing asymmetric cell divisions. This concept was very successful in describing the hierarchical organisation of tissues. However, experimental results on HSCs have led to the development of a concept that allows for more flexibility, the plasticity concept. According to this concept cells can loose and gain stem cell properties. If this applies to all possible states of a population, the states approach a stationary distribution which will be in general a broad distribution (Fig. 3b). In the following we will focus on models based on the plasticity concept.

The reversibility and stochasticity of cellular fate decisions has been studied by Loeffler and Roeder [54]. In their models [34, 73, 74] individual cells gain and loose stem cell properties depending on whether they localise inside or outside a specific niche environment, respectively. Thus, the environment directs the cellular fate and the reversibility of cell fate decisions is enabled by probabilistic switches between different micro-environments. The models were successfully applied to in vivo organisation of normal and malignant HSC populations.

However, MSC populations have been shown to expand while maintaining stem cell properties also in a homogenous environment. For modelling these systems we have expanded the ideas of Loeffler and Roeder by assuming that cells gain and loose stem cell properties according to a probabilistic process whose state-specific amplitudes are set by the environment. Within this approach cell fate decisions are basically reversible. The assumed fluctuations are hypothesised to be generated by intra- and extracellular noise triggering random transitions between different regulatory network activation patterns. This assumption is supported by experimental findings demonstrating that epigenetic gene silencing has a strong stochastic component [70, 96]. In the following we will give a brief description of a MSC population model that is based on this approach.

5 The Concept of Noise-Driven Differentiation

A growing body of evidence indicates that noise is not generally detrimental to biological systems but can be employed to generate genotypic, phenotypic, and behavioural diversity [44, 79, 80]. In particular, noise-driven solutions are expected to prevail in cellular adaptation to variable environments. Moreover, it has been proposed that biological systems have built-in molecular devices for noise control [1, 3, 28]. Together with the experimental results on MSCs, reviewed above, this has led us to suggest a model of noise-driven differentiation [37, 47], which will be introduced in the following.

5.1 General Assumption

In the model of noise-driven differentiation, cell differentiation is defined as loss of stem cell properties. It is quantified by a continuous state variable α that can adopt values between 0 (full stem cell competency) and 1 (fully differentiated cell).

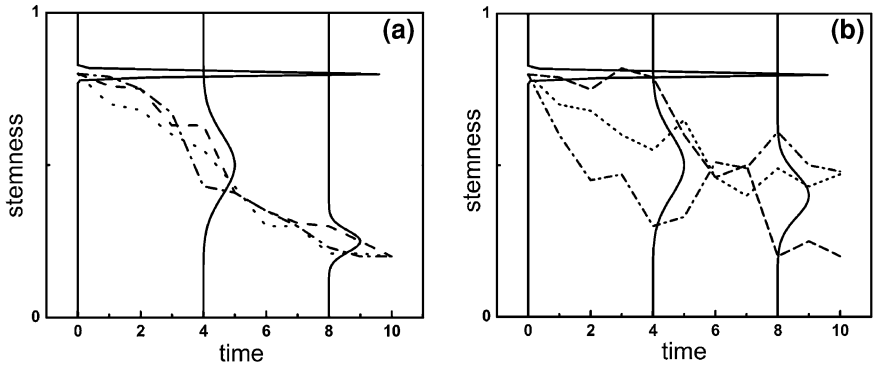

Fig. 3 Cell fate trajectories of individual cells according to different stem cell concepts. Cells of a defined differentiation state were selected (t = 0) and cultivated. **a** Pedigree concept: The cells loose stem cell properties due to a random process. Over time they accumulate again in a defined but more differentiated state. **b** Plasticity concept: The cells loose and gain stem cell properties in any state. The population approaches a stationary distribution over time. *Solid lines* denote sketches of the distribution of cell states at different time points. *Dashed lines* are trajectories of individual cells

Each value of α may represent a set of regulatory network activation patterns. From the molecular point of view, α may depend on abundance and subcellular localization of proteins and RNAs, as well as other types of signalling and metabolic molecules [50]. In general, cell differentiation is assumed to be reversible.

Each cell's α -value fluctuates randomly with a state-dependent noise amplitude $\sigma(\alpha)$ (Fig. 4a). From its current α-value a cell adopts a new value α' with a randomization rate R which may in general depend on α. We assume R to be constant. α' is drawn from a Gaussian distribution $p(\alpha'|\alpha)$, centred around α with standard deviation $\sigma(\alpha)$.

$$p(\alpha'|\alpha) \propto \exp(-(\alpha' - \alpha)^2/2\sigma^2(\alpha)) \tag{1}$$

The state dependence of $\sigma(\alpha)$ is assumed to be determined by the environment. We describe this dependency by:

$$\sigma(\alpha) = \sigma_0[1 - \alpha f(E)] \geq 0 \tag{2}$$

Here, σ_0 denotes the noise amplitude for stem cells, i.e. $\sigma(\alpha = 0)$. f(E) is a function describing the environmental impact. Positive fluctuation amplitudes require that $f(E) < 1$. In a simple approach it can be a constant $f(E) = f_0$. In this case $f_0 < 0$ describes stem cell supporting environments and $1 > f_0 > 0$ describes differentiating environments [37]. Differentiation is assumed to occur independently of cell proliferation as found in progenitor systems [8].

In contrast, cell proliferation is assumed to be differentiation state dependent. We assume that only cells in intermediate differentiation states with $\alpha_s < \alpha < \alpha_d$ proliferate (see Fig. 4b). For these states we assume an identical growth time τ.

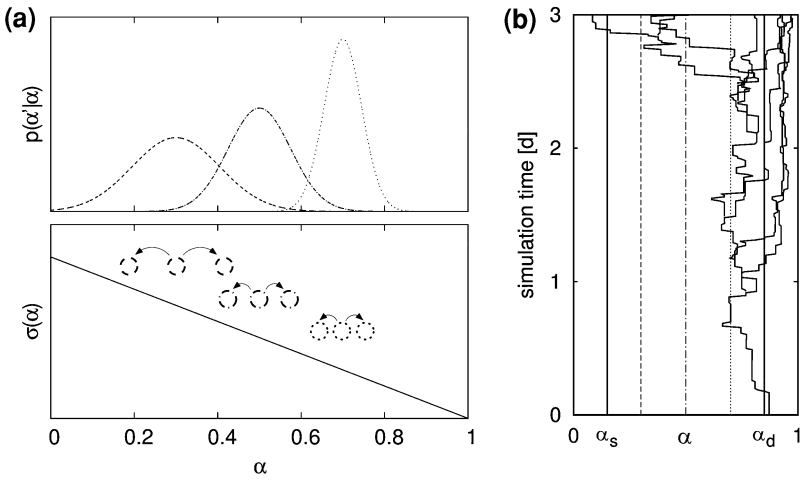

Fig. 4 Mechanisms of noise driven MSC differentiation. **a** Modelling fluctuations of the differentiation state α. *Upper panel*: Gaussian conditional probability function $p(\alpha'|\alpha)$ for the transition $\alpha \rightarrow \alpha'$ for $\alpha = 0.3$, 0.5 and 0.7. *Lower panel*: A decrease of the noise amplitude $\sigma(\alpha)$ with α results in an accumulation of cells at higher values of α. **b** A pedigree of a differentiated, quiescent cell ($\alpha > \alpha_d$) illustrating the model concept. After some time the cell regains proliferative capacity ($\alpha_s < \alpha < \alpha_d$) and generates a number of progeny. Moreover, there is a non vanishing probability of even regaining stem cell properties ($\alpha < \alpha_s$)

Stem cells ($\alpha < \alpha_s$) and differentiated cells ($\alpha > \alpha_d$) do not proliferate. During the growth process cells may frequently switch between proliferative and non-proliferative states. This will result in an effective cell growth time larger than τ.

The rate of randomization R is a model parameter that can be used for fitting experimental data. For MSCs it has been set to about 1 update per hour for a stem cell noise-amplitude of $\sigma_0 = 0.15$. Growth times are given by experimental observed minimal cell cycles times, which is about 11 h for MSC. The choice of α_s and α_d is somewhat arbitrary, as there is no clear phenotype related to it. We have chosen $\alpha_s = 0.15$ and $\alpha_d = 0.85$. Details can be found in Krinner et al. [47].

A straightforward method for quantifying the population heterogeneity is to calculate the normalised Shannon entropy from the distribution of the α states (here for a histogram of n bins):

$$H = \frac{-1}{\ln(n)} \sum_n p(\alpha_n) \ln(p(\alpha_n)) \tag{3}$$

Figure 5 shows the dependence of H on f(E) assuming a constant $f(E) = f_0$ between 0 and 1. H decreases with increasing f(E). However, the distribution remains heterogeneous (close to maximum entropy) as long as f(E) gets close to 1. These changes are much more pronounced in quiescent populations $1/\tau = 0$ compared to proliferating populations $1/\tau > 0$. Thus, very efficient noise control mechanisms are required in order to generate a homogeneous proliferating

population. It appears that this is given under artificial in vitro conditions, as e.g. high oxygen tension, leading to fast accumulation of cells in senescent states [101].

5.2 Exemplifying the Environmental Impact

A factor that strongly impacts MSC proliferation and differentiation is oxygen. It has been shown that MSC-derived cell populations show higher proliferative activity when cultured under low oxygen tension (2–5% pO_2) compared to high oxygen tension (20–21% pO_2) [19, 52, 71]. Moreover, cell populations expanded at low oxygen tension show a faster and more directed differentiation into osteoblasts, adipocytes [35, 52] and chondrocytes [59, 97]. As shown in Fig. 5d, our model predicts that increased proliferation increases the population heterogeneity. This would suggest describing the oxygen dependence in terms of the growth rate, i.e. by assuming a higher growth rate $1/\tau$ at low oxygen tension. However, in case of a proliferation stop, e.g. according to contact inhibition of growth (see below), cells at 5% pO_2 would acquire a senescent state as fast as at 20% pO_2, which has not been observed. Thus, we decided to model the observed effects of oxygen tension by changes of the noise-profile exclusively.

Actually, we assumed that cell adaptation to non-physiological high oxygen tension results in decreased state fluctuations which subsequently lead to an accumulation of cells in differentiated states. In contrast, low oxygen tension conserves stem cell and progenitor states by enabling high amplitudes of the state fluctuation. We described this dependency by a Hill function $f(pO_2/pO_2^{max})$ approaching 0 and 1 at low and high pO_2, respectively:

$$\sigma(\alpha) = \sigma_0\left[1 - \alpha f\left(pO_2/pO_2^{\max}\right)\right] \text{ with } f(X) = \frac{X^n}{X^n + K^n} \tag{4}$$

The related 'noise landscape' is shown in Fig. 6 together with the differentiation state profiles for 5% and 20% pO_2. The parameters of the profiles were obtained by reproducing the clonal growth properties at these conditions [47]. Using these distributions as input we were able to simulate the experimentally observed impact of low oxygen expansion on subsequent differentiation in pellet culture which appears to be a result of limited lineage plasticity of MSC populations.

5.3 Impact of Cell−Cell Interactions (Contact Inhibition of Growth)

The above assumptions exclusively consider intrinsic regulation of the MSC state. The impact of cell-cell interaction has been neglected so far. However, it is well known that MSC expansion, lineage specification and terminal differentiation

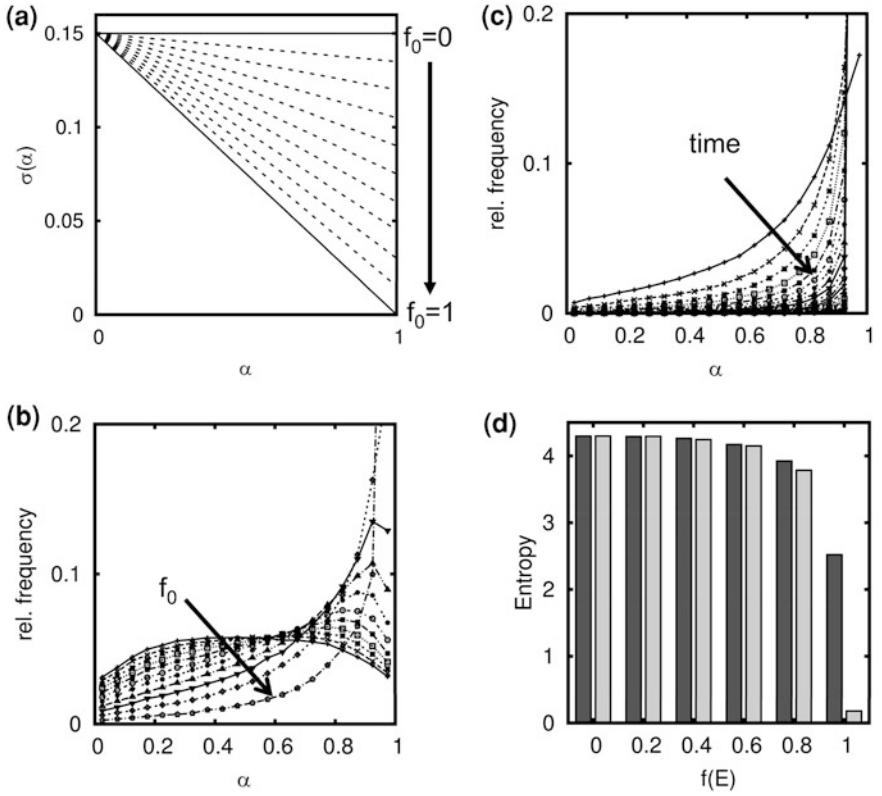

Fig. 5 Simulation results on noise driven MSC differentiation. **a** Noise-profiles with monotonously increasing f(E): $0 < f(E) = f_0 < 1$ were applied. **b** The calculated differentiation profiles of the MSCs demonstrate accumulation of the cells in differentiated states $\alpha > \alpha_d$. The amount of differentiated cells increases with f_0. **c** Equilibration of the profiles occurs on the scale of a few days. Shown are snapshots of a fast equilibrating system with $1/\tau = 0$ after switching f(E) from 0 to 1 (time steps $\Delta t = 3$ h). Eventually all cells accumulate in the fully differentiated state (bin: $0.95 < \alpha < 1.00$). Except for the first profile at 3h (+) its fraction exceeds 0.2. **d** The normalised entropy of the α-distributions strongly decreases if f(E) gets close to one. For $1/\tau = 11$ h (*dark grey*) the systems equilibrate as shown in **b**. For $1/\tau = 0$ (*light grey*) the tendency to differentiate is more pronounced and more homogeneous populations are formed

depend or even require such interactions [93]. A well known control mechanism of proliferation based on cell-cell interaction is contact inhibition of growth. According to this mechanism cells that form close contacts stop proliferation. Preventing this phenomenon by applying sophisticated culture conditions can significantly enlarge cell culture harvest [57].

In order to consider such regulation individual cell-based models of cell populations have been established [26, 32, 33]. In these models each individual cell is described by a physical object that can deform, adhere to other cells or a

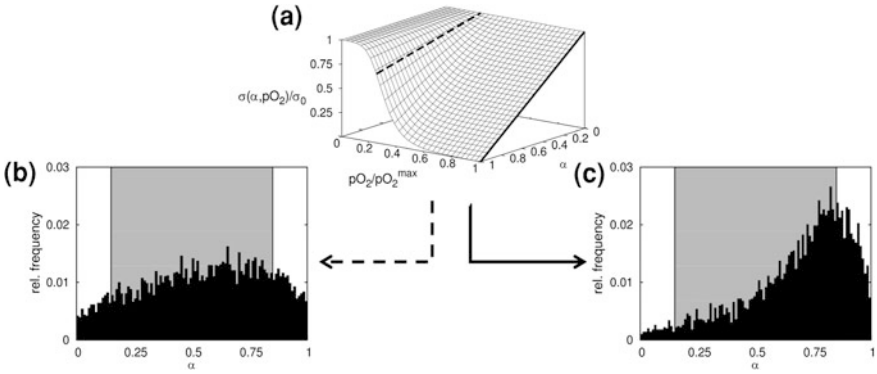

Fig. 6 Noise-landscapes of MSCs in dependence of the oxygen tension. **a** A low oxygen environment (5% pO_2) is characterised by high noise amplitudes in all states (*dashed line*), while a high oxygen environment (20% pO_2) is characterised by low amplitudes in high α-states (*solid line*). **b**, **c** The α-profiles of the low and high oxygen environments for $\tau = 11$ h

substrate, can move, grow and divide. Cell proliferation is modelled assuming a two phase cell cycle where during the interphase, a cell doubles its volume by stochastic increments and during the mitotic phase, a cell divides into two daughter cells of equal properties. Thereby, cell shape is often approximated by a sphere (see Fig. 7a) and the elastic deformation of a cell subject to compression by other cells or substrate is modelled by the Hertz-Model [49]. Contact inhibition of growth is considered by these models assuming that a cell stops growth if the sum of contact forces on it exceeds a critical threshold value.

Simulating monoclonal expansion by applying such an individual cell-based model one observes a specific distribution of proliferating cells within the growing clone [26, 32], with quiescent cells located in the core and proliferating cells at the periphery of the clone. In such simulations one can simply record the pedigree of the clone and can calculate the spatial distribution of the different generations throughout it. Thereby, the generation numbers are uniquely defined for all cells by the recursion $m_l = m_m = m_k + 1$ for the daughter cells l and m of mother cell k; i.e. m counts for the number of divisions that has been carried out until the cell was born. The result of such a simulation is shown in Fig. 7c. One observes a heterogeneous distribution with 'younger' cells in the core and 'older' cells at the periphery. Within about 12 days differences in 'age' of more than ten generation have been established.

Obviously, MSC shape is very different from being spherical-like. Considering that the cell−cell interactions resulting in contact inhibition of growth depend on the cell shape, the reliability of simulation results, as shown in the top row of Fig. 7, seems to be questionable. We therefore developed a more sophisticated model of MSCs that explicitly accounts for podia formation [38]. This approach builds on the model assuming spherical cell bodies. In addition, cells feature podia that generate forces for cell spreading and movement. Podia of model cells retract

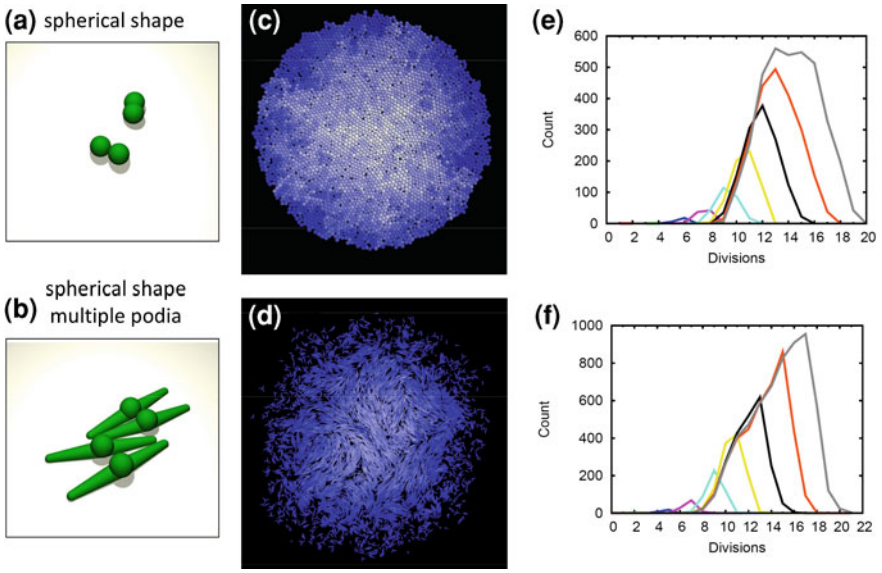

Fig. 7 Formation of an age distribution within expanding MSC clones. Comparison of a simple model assuming spherical cell shape (**a**, **c**, **e**) with an advanced model assuming that cells do form multiple podia (**b**, **d**, **f**). **a**, **b** Cell shape of the respective model. **c**, **d** Due to active contact inhibition the growing cells in the centre of the populations stop proliferation. Accordingly, a gradient in generation number is formed, here indicated by colour saturation. *White* cells are of generation 8, and *dark blue* cells of generation 22. **e**, **f** Development of the distribution of generation numbers in a growing colony over 8 days (*blue*: d2, *magenta*: d3, *cyan*: d4, *yellow*: d5, *black*: d6, *orange*: d7, *grey*: d8). While the formation of an age gradient is a generic phenomenon, the details of the distribution of generation numbers depend on the biophysical model.

prior to cell division and align to each other as is also experimentally observed in proliferating MSCs in vitro (see Fig. 1b, [15]). In this model cell migration is accomplished by protrusion and traction forces exerted by model podia. The number of podia is dynamically controlled by adaptation of the probabilities of podium generation and inactivation. The migration phenotype largely differs between cells with only one active podium (mostly ballistic movement with random turns) and cells with multiple active podia (mostly stretched out and resting with random reorientation moves). Details can be found in Hoffmann et al. [38].

Simulating monoclonal growth applying this podia model one observes results comparable to those observed in the simple model. In particular this regards the spatial distribution of cell generations (see Fig. 7d). However, quantitative differences can be observed depending on the choice of the model parameters, which include parameters determining cell friction forces, podia lengths and activation/deactivation rates as well as the parameters defining the sensitivity of the cells to contact inhibition. Regardless of these differences the age structure appears to be a generic feature of growing cell populations. Nevertheless, it has been not considered in MSC model approaches so far.

6 The Case of Stable, Inherited Heterogeneity: MSC Ageing

In order to define age dependent properties of the MSC populations we extended our former approach and assumed that stem cell states become de-stabilised with 'cellular age' resulting in an increased tendency for spontaneous differentiation. We model this scenario assuming that the noise amplitude of a cell $\sigma(\alpha)$ depends on its generation number m. Accordingly, each individual cell is characterised by its noise amplitude, which depends on the cell's differentiation state α and age m:

$$\sigma(\alpha, m) = \sigma_0[1 - \alpha f(E)] + m\, r_D[1 - 2\alpha] \geq 0. \tag{5}$$

The first term on the right hand side of Eq. 5 defines the extrinsic, environmentally determined noise amplitude (see Eq. 2). Here, σ_0 denotes the noise amplitude for initial stem cells, i.e. $\sigma(\alpha = 0,\ m = 0)$. The second term on the right hand side defines the effect of ageing, which is most obvious considering the noise amplitudes in stem cell states $\sigma(\alpha = 0)$:

$$\sigma(\alpha = 0) = \sigma_0 + m r_D. \tag{6a}$$

This stem cell noise amplitude increases with each generation by the rate r_D. This assumption allows us to quantify the 'age' of a cell by its stem cell noise amplitude $\sigma(\alpha = 0)$. The noise amplitude of differentiated cells is given by:

$$\sigma(\alpha = 1) = \sigma_0[1 - f(E)] - m r_D > 0 \tag{6b}$$

This amplitude decreases with m. For $m_{RE} = \sigma_0[1 - f(E)]/r_D$ it is equal to zero. In order to ensure that $\sigma(\alpha = 1) > 0$, we assume that σ becomes independent of m for $m > m_{RE}$.

According to these assumptions the heterogeneity of a population can be described by the probability distribution to find a cell of age m and in state α. During expansion the average number of cell divisions grows, thus noise amplitudes in differentiated states decrease and, consequently, cells accumulate in these states. This effect is independent of the environment. It is determined by the ageing rate r_D. This rate determines also the age m_{RE} of replicative senescence. Assuming $r_D \sim 2.5 \times 10^{-3}$ one obtains an upper limit of m_{RE} of about 60. This can be seen as an upper bound to the experimental findings so far (<30 at 20% pO_2, [95] and <40 at 3% pO_2, [30]).

In simulations of the model we assumed for the environmental term of the noise amplitude:

$$f(E) = 2\ (1 - \sigma_E/\sigma_0), \tag{7}$$

where σ_E is the mean noise amplitude defined by the environment.

Figure 8 shows results on the simulated age-structure and population heterogeneity applying the age model and assuming $\sigma_e = \sigma_0 = 0.075$. After 20 days of

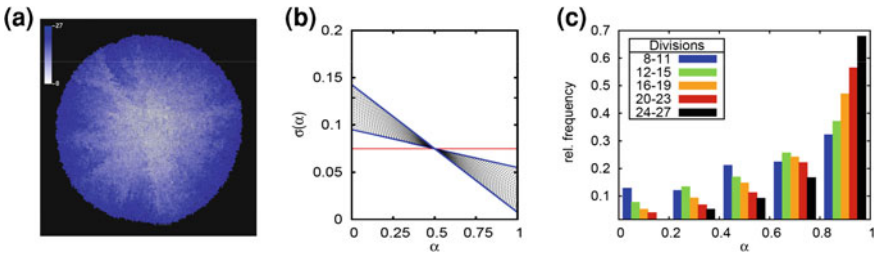

Fig. 8 Simulation results applying the ageing-model. **a** The observed distribution of the generations is comparable to that observed without ageing. **b** Age-dependent noise profiles as set before (*red*) and observed after expansion (*grey*). A considerable heterogeneity regarding age has developed. **c** Distribution of differentiation states depending on the age. Young cells (*blue*, $m = 8–11$) include a relevant fraction of undifferentiated cells with $\alpha < 0.2$, while most of the old cells (*black*, $m = 24–27$) reside in a differentiation state of $\alpha > 0.8$

expansion a few cells of the population have nearly reached $\sigma(\alpha = 0) = 0.15$ as assumed in the other applications. However, a large heterogeneity regarding age has been established ($7 < m < 28$). With increasing age the cells tend to accumulate more and more in differentiated, senescent states (here, $\alpha > 0.8$). This is in nice agreement with experimental observations on ageing MSCs [45, 95].

As found in our simulation studies comparing MSCs that were expanded at high and low oxygen tension (Sect. 5.2, [47]), these differences between young and old cells in the distribution of the differentiation states will also manifest in differences in their lineage specification and functional differentiation potential. Accordingly, aged cells are characterised by a lower regenerative potential compared to young cells. This is again in agreement with experimental findings [45, 95].

In summary the model is capable of describing experimental findings on the environmental dependent MSC ageing. The underlying concept is general in the sense that in vitro and in vivo ageing can be assumed to base on the same principles. This is supported by recent experimental findings demonstrating that ageing and replicative senescence have related effects on stem cells [94] .

7 Discussion

Grafting of MSCs is an emerging technology to repair tissues and organs of mesenchymal origin. A prerequisite of these applications is rapid and massive MSC expansion. This becomes obvious considering the isolation process of these cells. Bone marrow is an important source of MSCs from where they can be easily isolated and purified. However, only about 0.01% of nucleated bone marrow cells carry MSC properties. Accordingly from a 20 ml aspirate usually only up to 50.000 MSCs are obtained [6]. Consequently, at least 5PDs are required in order to obtain the 10^6–10^7 cells that are required in a typical application.

While even the isolated populations are heterogeneous regarding different functional aspects, this heterogeneity further evolves during amplification. Thereby the actual changes depend on the details of the culture conditions which will essentially impact the cell harvest. Thus, to understand the origin and development of MSC heterogeneity will help to improve their cell culture conditions. Moreover, it represents a basic step towards individualised therapies [31].

We here presented a mathematical approach to MSC populations which allows considering each cell individually. Thus, the approach enables us to simulate the changes in the population behaviour based on changes in single cells which are assumed to depend on their particular regulatory state. Our model allows simulations to closely follow standard in vitro expansion and differentiation protocols of MSCs such that they may be viewed as experiments 'in silico' (on the computer) and the results can directly be compared to those found from experiments.

The approach presented here assumes that noise is predominant in most cellular states. Its essence is that MSC population structures are determined by state-specific noise [37] forming a 'noise landscape', where low noise states represent the attractor states. Cells subjected to an environment not matching their internal state are assumed to be destabilised by a high noise amplitude. They subsequently adapt to this environment by travelling towards low noise states. Extensions of the proposed noise-driven approach to lineage specification and functional differentiation have been described [47]. Thereby, decision making in individual cells during these processes was linked to particular differentiation states and cell–cell interactions. In particular, lineage specification was assumed to require sufficiently high stemness, i.e. α values below a certain threshold. In this application MSC ageing was not considered. However, while changing the distribution of the differentiation states, obviously ageing will also affect the lineage specification dynamics, with aged cells rarely or never able to switch lineage. Accordingly, we expect also functional differentiation to be hampered.

A question not raised so far is whether there is a functional relevance of MSC heterogeneity regarding expansion and differentiation. The observed heterogeneity of MSCs could actually represent a functional aspect of their organisation. For example high proliferation potential of a subpopulation could originate from secretory properties of another subpopulation in the close neighbourhood. In a recent study Krinner et al. analysed the expansion and differentiation properties of single cell derived clones and compared them with the properties of their mother clone (unpublished results). As the high expansion potential of individual clones outcompetes that of their mother population, they were not able to provide any evidence for a specification of subclones into fast expanding clones and expansion supporting secretory clones.

In order to get deeper insights into MSC organisation a detailed characterisation of the microenvironments in which MSCs reside in vivo is required. Effective methods for screening MSC properties in complex in vitro environments have been already developed [55, 56]. Moreover, regeneration experiments on primary MSCs as suggested in Krinner et al. [46] will help to understand MSC adaptation to changing environments and to identify properties that are inherited. This would

also help to solve the question whether proliferation alone (as assumed in the models presented) or/and adaptation to changing environments is a source of persistent clonal differences. For this purpose FACS analysis of selected markers should be combined with gene expression analysis as demonstrated by Chang and co-workers [10]. Moreover, time-lapse online analysis of the expression of stemness and differentiation markers by individual MSCs could not only provide direct evidence for the assumptions made in the noise-driven differentiation model, it would also delineate the emerging structure of noise-landscapes.

The proposed model is capable of explaining an entire panel of experimental observations regarding MSC heterogeneity. However, the molecular basis of the assumed noise-profiles and their dependence on the differentiation states, the environment and age remain speculative. Models have been suggested that involve e.g. Wnt-pathway activity [3]. A general model framework linking noise-landscapes to the dynamics of regulatory networks is missing. Thus, building up multi-scale models that bridge the current gap between the increasing amount of molecular data and observed cellular phenotypes represents a current need in order to improve our understanding of the heterogeneity of MSCs in vitro. Their validation will require sophisticated experimental studies on the single cell level.

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