Proceedings of the 2nd International Conference on Modern Medicine and Global Health
DOI: 10.54254/2753-8818/35/20240930

The stochasticity in organismal development: Stem cell heterogeneity

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Abstract. Organismal development was traditionally believed to be a tightly regulated process; however, recent discoveries have uncovered its underlying stochasticity. Numerous questions remain unsolved regarding the effect of stochasticity, the origin of variability, to what extent stochasticity influences development, and how the balance between randomness and robustness is maintained. This dissertation provides an overview of beneficial and detrimental aspects of stochastic events in the organismal development process, with a particular focus on explaining how intrinsic and extrinsic noise contribute to stem cell heterogeneity, which plays a crucial role in their differentiation and self-renewal. Furthermore, the current research limitations and significance of future exploration in this field were highlighted in the end.

Keywords: organismal development, stochasticity, stem cell heterogeneity, intrinsic and extrinsic noise.

1. Introduction

Organismal development is a complex process characterized by a series of orchestrated events that transform a single, naïve cell into multicellular tissue and ultimately into a mature adult. Previously, development has been heavily studied and considered as a well-designed, deterministic course of events, supported by evidence including homeotic genes which can determine the development patterns of body segments in drosophila, and morphogen gradient like Bicoid which establishes the body axis in drosophila embryo. However, recent studies have started to shed light on the significance of stochasticity in organismal development which has been thought to be equally important as deterministic factors. Stochasticity arises from varied sources including cellular noise, environmental variability, and gene expression fluctuation. Typical stochastic examples include viral life cycle decision-making, the bet-hedging mechanism in bacteria and eukaryotic cell fate determination.

This dissertation aims to investigate the role and origin of stochasticity in organismal development with a specific focus on the stochastic nature of stem cell (SC) heterogeneity. By exploring the stochastic events in SCs, we can have a better understanding of mechanisms which underlie their differentiation and self-renewal, and ultimately the overall developmental processes. Additionally, we will review the recent technological progress and identify the prospective areas for research in this field.

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2. Dual Perspectives of Stochasticity

Stochasticity can be observed universally in microbial, single-cell eukaryotes and multicellular organisms which have development processes. The impact of stochasticity on organismal development has both advantageous and detrimental aspects. On one hand, it enhances the adaptability of organisms by optimising their fitness in response to environmental changes. However, on the other hand, it can also promote the progression of diseases and ageing processes.

2.1. Benefits of stochasticity

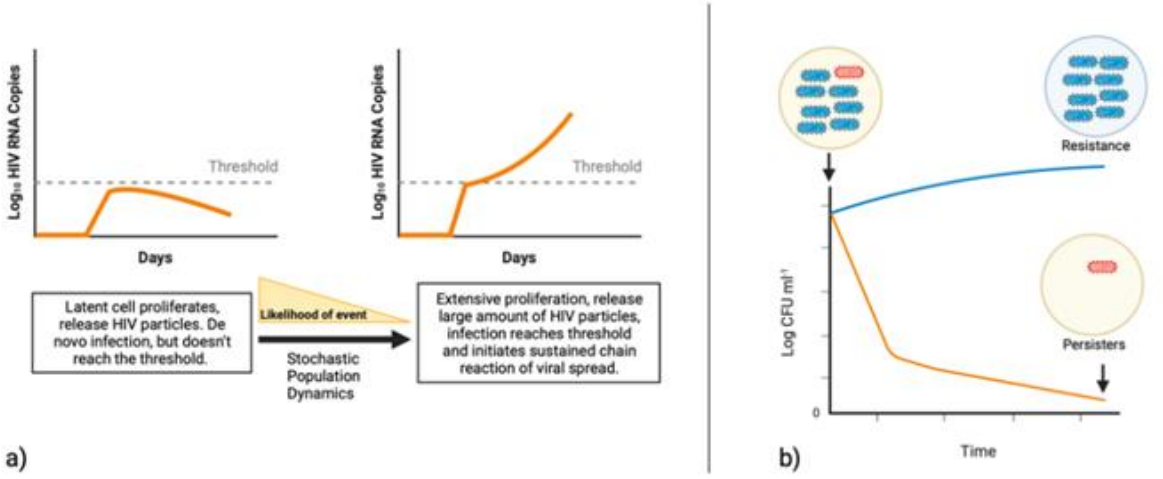

Figure 1. Stochastic model in HIV and bacteria. a) Stochastic transition to exponential HIV growth. The transition from latent to exponential growth state occurs when the initial virus release exceeds the critical growth threshold. The exponential growth state is rarer compared to the latent state; b) Growth pattern of antibiotics treatment of resistant bacteria and persisters. After the initial addition of antibiotics to a heterogeneous population of bacteria (yellow), the susceptible bacteria will be killed rapidly followed by a slower decrease in the colony forming units (CFU) as persisters (red) which occupy a small fraction of the population will survive (orange curve); With the addition of antibiotics to a homogeneous population of resistant bacteria (blue), there will be a continuous growth of bacteria population. Additionally, it is believed that SC population heterogeneity including variation in SC self-renewal and fate determination in an isogenic clone can be caused by both random intrinsic and extrinsic noise factors. This variability is crucial as it creates diversity in cellular functions as well as provides organisms with developmental plasticity and regenerative capabilities. Later sections will provide more detailed information on this topic.

Viral life cycle decision-making is a highly stochastic event providing numerous advantages for viral survival. These include enhanced viral adaptability, expanded host range, and evasion of the host immune response. Human immunodeficiency virus (HIV) is one of the most well-known examples. HIV enters a latent state by integrating into the genome of memory $CD4^+$ T lymphocytes as a provirus, thereby establishing a reservoir population which can persist for a long period of time. However, these latent viruses can be reactivated and trigger the exponential growth phase in which a large amount of virion is produced, leading to cell lysis. The state switching from latency to exponential HIV growth only occurs when the fluctuating viral release amount meets the critical threshold (Figure. 1a) [1]. This population dynamics can be caused by factors including the fluctuation in viral gene expression and the host immune environment [2]. Bet-hedging is an evolutionary strategy which allows organisms to increase their survivability in fluctuating environmental conditions by diverging into different phenotypes and sacrificing the optimum state. This model has been well studied on prokaryotes:

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evidence suggests that when an isogenic population of bacteria is treated with sufficiently strong antibiotics, most bacteria will die while a subpopulation known as persisters will survive. Unlike resistant bacteria which are always insensitive to antibiotics, persistent bacteria remain susceptible even after removal of antibiotics and population recovery (Figure 1b). The presence of persisters is linked to the heterogeneity in the original bacteria population, arising from stochastic phenotypic switching between the normal state and slow-growing persistence state [3].

2.2. Downside of stochasticity

Although stochasticity is critical in organismal development and adaptation, it also has deleterious aspects which need to be minimized to maintain robust functionality. This is supported by evidence such as the low variability of housekeeping genes, which suggests an intentional effort to minimize randomness. Gene regulatory networks (GRNs) are also organized to minimize stochasticity and maintain precise control over the gene expression [4]. Stochasticity was found as a causative and promoting factor in many diseases. For instance, cancer cells can exist in varied phenotypic states even though they are derived from the same tumour tissue. This heterogeneity arises from stochastic cellular state transitions caused by intrinsic factors (e.g. random genetic mutations) and extrinsic factors (e.g. tumour microenvironment) [5]. For example, the dynamic expression of some drug-induced proteins can lead to varied drug responsiveness among cells in the cancer cell colony [6]. These differences provide the foundation for selectable variants required by Darwinian evolution processes and are vital for the tumour evolution [7]. Furthermore, studies on breast tumour cells have demonstrated that the cancer cell states are interconvertible, following the Markov model—cells convert stochastically between states and the cell transition only depends on the current cell state and is irrelevant to their past states [8]. Apart from cancer, stochasticity is also closely related to ageing processes. Accumulation of DNA damage and mutations over time is considered a main contributor to ageing. According to a previous study, gene expression noise is present in young mouse cardiac muscle, but it significantly increases in older mouse hearts. Researchers further induced transcription noise by treating mouse embryonic fibroblast with hydrogen peroxide and the result suggests that this increased gene expression variability can cause higher levels of genome damage and ultimately contribute to the ageing progression. [9].

3. Stochastic Nature of SC Heterogeneity

In the previous chapters, we have explored the multifaceted roles of stochasticity and discussed its benefits and drawbacks. SCs play a crucial role in organismal development and possess exceptional regenerative capabilities. Understanding the stochastic nature of SCs and the origin of this randomness will be vital for the advancement of regenerative medicine and the treatment of diseases. SCs are cells under the undifferentiation state and able to self-renewal or develop into specific cell types. SCs can be classified according to their origins and differentiation potentials: Embryonic SCs (ESCs) are derived from the early-stage embryo and is highly pluripotent which means they have no predetermined program and can differentiate into all types of cells in the body. Somatic SCs like hematopoietic SCs (HSCs) have more limited differentiation potential than ESCs and their main function is replacement and regeneration. The differentiated cells can also be reprogrammed into induced pluripotent SCs (iPSCs) through varied defined factors [10]. The population of SCs carries inherent heterogeneity. By utilizing the single-cell droplet-barcoding RNA sequencing technique, Klein *et al* illustrated that the ESCs population is highly heterogeneous and shows a dynamic differentiation pattern [11]. HSCs in a cell clone are proven not equipotent as well and have a biased differentiation towards different lineages [12]. The heterogeneity within the SC population is also a bet-hedging strategy which allows SCs to respond and adapt to changing developmental stages and this cell-cell variation arises from a combination of intrinsic factors and extrinsic factors [13].

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3.1. Intrinsic factor

The intrinsic randomness of molecular dynamics and intracellular interactions can give rise to inherent stochasticity. This stochasticity manifests as fluctuation in gene expression, often referred to as "gene noise", and plays a significant role in multiple biochemical processes including transcription, translation, and epigenetic modifications which ultimately contribute to cellular heterogeneity.

3.1.1. Transcription factor heterogeneity. Transcription factor heterogeneity is a typical intrinsic factor that contributes to the SC variation. In the ESC model, multiple transcription factors have been proven to express heterogeneously, including *Klf4* and *Tbx3* [14]. One of the most extensively studied examples is NANOG which is a homeodomain transcription factor and a core member of the transcriptional regulator network (TRN) of pluripotent SCs (PSCs). The NANOG-dependent feedback loops not only determine the fate of ESCs but also maintain their self-renewal ability and pluripotency [15][16]. Interestingly, even with the permanent deletion of the *Nanog* gene, ESCs can still retain their self-renewal ability due to the presence and functioning of other important feedback loops. This indicates that NANOG does not solely determine the commitment, but ESCs are more prone to differentiate with low NANOG levels (typically 5%-20% of the cell population has a low Nanog-level state) which highlights the pluripotency safeguarding role of the NANOG [16]. By utilising fluorescent reporters to investigate temporal dynamics of *Nanog* expression in mouse ESCs (mESCs), scientists illustrated that low-NANOG (LN) and high-NANOG level (HN) can continuously interconvert. This heterogeneous *Nanog* expression is widespread in mESCs population which provides SCs with opportunities to explore different lineage options and increase the probability of transitioning to alternative functional states [17]. How does stochastic gene expression arise? Two potential underlying factors are transcriptional bursting and allelic switching. Transcriptional bursting is one of the main sources of intrinsic noise in ESCs which contributes to around 45% of the total variation. Through quantitative analysis of *Nanog* transcription, an infrequent, pulsatile and stochastic pattern was observed [18]. This pulsatile transcription, also known as transcription bursting originates from multiple factors (Figure. 2a). The chromatin-based model suggests that promoter activation is dependent on the relatively slow dynamics of chromosomes, where nucleosomes compete with transcription factors upstream of the transcription start site and this competition will result in a random transcription pattern [19]. Histone modification also plays a crucial role here. For example, histone acetylation promotes the activation of RNA pol II and can regulate bursting frequency-mediated changes. The availability of TF is another proposed factor, while the underlying mechanism remains unclear. One related finding involved the use of live cell super-resolution techniques, which showed that the binding site of transcriptional factors (TF) at the target motif may be distinct from the active transcriptional site, as evidenced by the case of clustering of TF SOX2 to trigger the expression of *Oct4* genes. Finally, cis-regulatory sequences including both distal enhancers and proximal promoters can regulate transcriptional bursting mainly by modulating the bursting frequency [20]. The combination of the aforementioned points contributes to the fluctuating gene expression observed. To determine the fluctuating transcriptional states during bursting, multiple models have been suggested (Figure 2b). The first and simplest model was the Poisson model which describes a fixed transcriptional activity, while it is not convincing enough to explain the complicated dynamics of transcription observed. Therefore, the two-state model is the most widely accepted. It demonstrates two discrete states (active and inactive) of genes, with only genes in the active state being expressed, the random turning on or off the genes allows the pulsatile expression [21]. More complicated models have also been proposed recently, including three-state, multi-state, continuous and refractory models, even though the detailed mechanism behind these models remains unclear [22].

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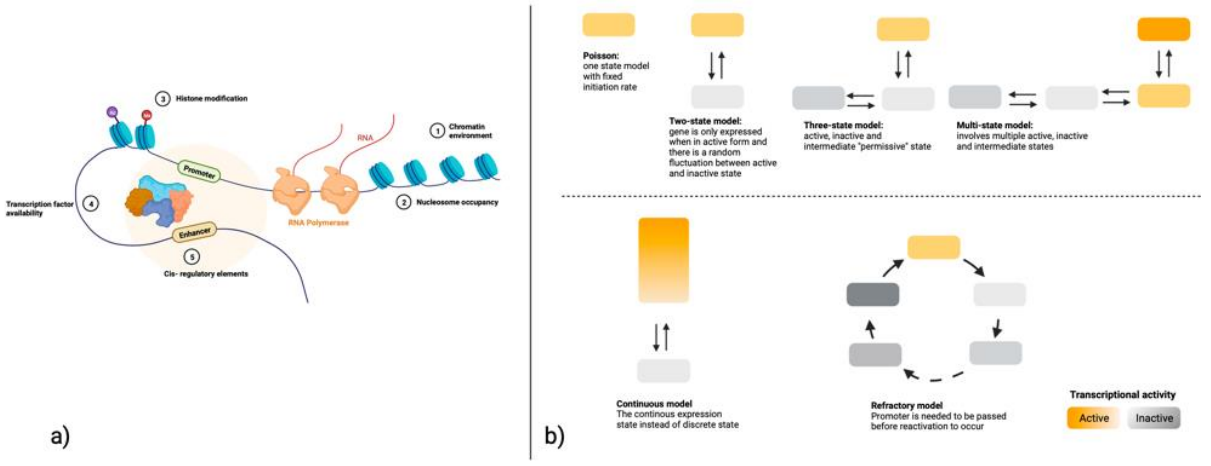

Figure 2. Overview of mechanism and model of transcriptional bursting. a) Factors affect transcriptional bursting; b) unified models of transcriptional bursting dynamics. Active (orange) and inactive (grey) transcriptional states are shown.

Allelic switching is another factor that contributes to dynamic gene expression. By utilizing RNA fluorescence in situ hybridization (RNA-FISH), it has been discovered that around 59% of ESCs exhibit NANOG transcriptional firing. Within this population, 14% fire from both alleles while 45% fire from a single allele. This indicates that under most conditions, ESCs prefer firing one allele instead of both (Figure.3a). More interestingly, previous studies have illustrated that if allele switching occurs on a rapid timescale compared to the lifespan of protein and mRNA, a high transcription rate can be observed for both *Nanog* alleles, while if the switching speed is slow, *Nanog* mRNA concentration will decrease (Figure.3b). Therefore, there will be a discrepancy between allelic firing and mRNA transcripts generation when the frequency and strength of allelic firing and switching are irregular [22].

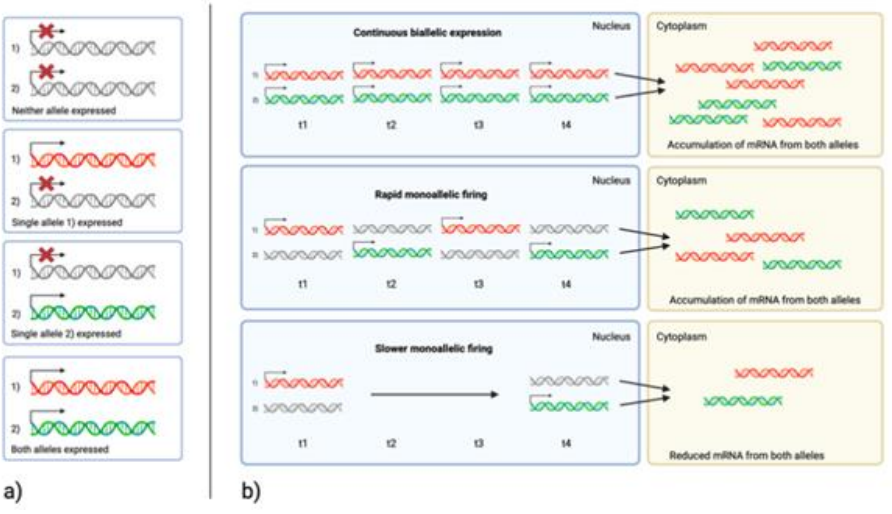

Figure 3. The allelic switching mechanism of *Nanog*. a) *Nanog* allele expression has four different states: neither allele expressed, single allele expressed (allele 1 and 2 respectively) and both alleles expressed. The variation of the expression pattern shows that transcriptional firing can contribute to the heterogeneity of ESC population; b) When both *Nanog* alleles (red and green) are expressed continuously, transcripts of both alleles will accumulate and be translated into NANOG protein later; in case of monoallelic firing, When the rate of firing is quick, it still leads to the accumulation of mRNA from both alleles, while if the rate is slow, the concentration of mRNA from each allele will decrease

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The normal function of NANOG is highly interdependent with other regulatory factors including OCT4 and SOX2, these three factors interconnect with each other to form the SON (SOX2, OCT4, NANOG) network. OCT4 is a key transcription factor restricted to the pluripotent and germ-line cells and SOX2 is a DNA-binding protein which synergistically interacts with OCT4 by sharing the same binding site (Figure 4a), the OCT4-SOX2 complex can act as a heterodimer regulating genes including *Nanog*, *Sox2* and *Oct4* (Figure 4b). Conclusively, the interactions within the SON network and inherent transcriptional noise lead to the fluctuation of *Nanog* expression and contribute to the ESC population heterogeneity [23].

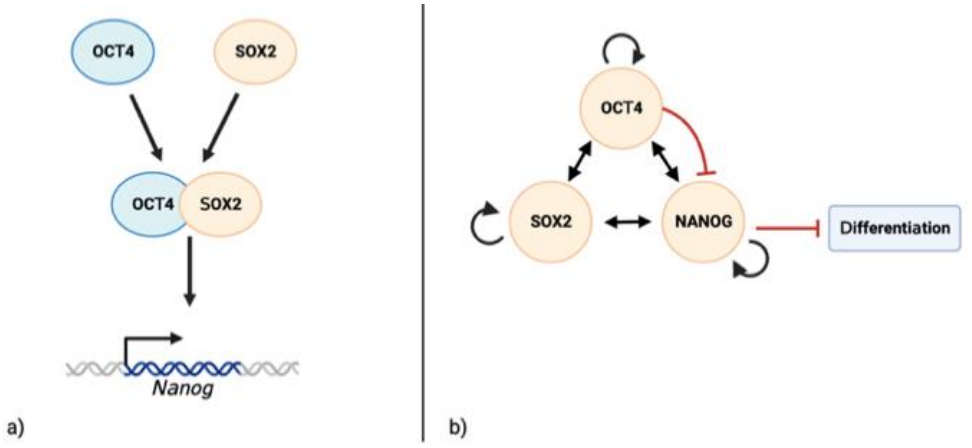

Figure 4. Overview of SON interaction**.** a) OCT4 and SOX2 are two critical TFs. *Nanog* gene expression is regulated by OCT4/SOX2 complex; b) SOX2, OCT4 and NANOG proteins interact with each other. NANOG protein derived from the expression of the *Nanog* gene can promote the expression of both the *Oct4* and *Nanog* gene, while it inhibits ESC differentiation. OCT4 protein can both inhibit and promote the expression of the *Nanog* gene and it also promotes *Oct4* expression as a positive feedback loop; SOX2 protein can promote the expression of *Nanog*, *Oct4* and *Sox2*.

3.1.2. Cell cycle variability. In addition to transcription factor heterogeneity, cell cycle variability was also found related to ESC fate decision-making. SCs in the S/G2/M phase actively and independently promote the pluripotent state, while they are more prone to differentiate in the G1 phase, which suggests G1 is a vital window for SC state transition under differentiation cues [24]. The absolute length of the G1 phase also influences SC differentiation, for instance, cells in the short G1 phase are more likely to differentiate into mesendoderm lineage while the long G1 phase allows cells to differentiate into neuroectoderm. Consequently, the variation in single-cell G1 length which operates in a dynamic equilibrium contributes to the heterogeneity in differentiation propensity within the PSC population [25]. With the help of the FUCCI reporter system, scientists discovered the role of cell-cycle regulator cyclin D in controlling cell differentiation signals and cyclin D-CDK4/6 complex plays a crucial role in restricting the nuclear localization of some important signalling molecules including Smad2/3. The dynamic expression of cyclin D proteins in the G1 phase provides a possible explanation for the observed variation in differentiation capacity among human ESCs alongside the progression of the G1 [26].

3.2. Extrinsic factors

Despite significant advancements that have been achieved in understanding the contribution of intrinsic factors to stochasticity, a considerable number of uncertainties remain which shows that hidden extrinsic factors are involved as well. Many studies have shown that stem cell fate is controlled by their specialized environment, also known as the stem cell niche [27]. Stem cell niche influences SC development through various pathways including secreted factors, cell-cell, or cell-extracellular matrix (ECM) interaction mitochondrial variability and environmental epigenetics (Figure. 5).

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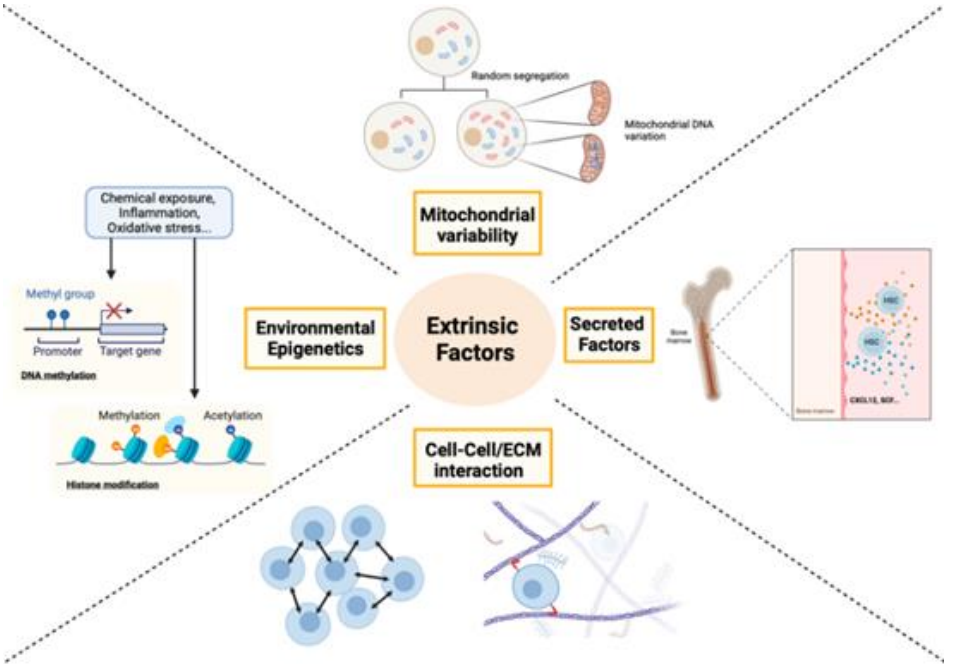

Figure 5. Extrinsic factors can lead to SC heterogeneity. Four main extrinsic factors cause SC heterogeneity. Firstly, mitochondrial variability, including both number and functional differences results in the cell-cell variation. Additionally, secreted factors (e.g. CXCL12 and SCF) released from bone marrow can alter the HSC niche thereby impacting HSC self-renewal and differentiation ability. Furthermore, cell-cell and cell-ECM interactions involving cell signalling are crucial determinants of SC development. Finally, Environmental cues including chemical exposure and inflammation can induce epigenetic modification which contributes to cell-cell variability as well.

The stem cell niche releases various factors including growth factors, morphogens, and chemokines. These secreted factors primarily prevent the death of lineage-committed progenitors, which arises stochastically. The interaction between secreted factors and the niche is highlighted in the case of haematopoietic SCs (HSCs). Within the HSC niche, paracrine secreted factors include thrombopoietin, C-X-C motif chemokine ligand (CXCL)-12 and SC factor (SCF) play a crucial role in maintaining the quiescence state and the self-renewal ability of HSC and the fluctuating secretion of these factors helps regulate the HSC stages progression [28]. Additionally, inflammatory stimuli can activate HSCs, promoting their proliferation and differentiation towards myeloid lineage. Additionally, inflammation is able to alter the niche environment which promotes the adaptability of HSCs under emergency myelopoiesis condition [29]. Apart from secreted factors, cell-cell interaction also regulates cell fate determination. By utilizing different microenvironments to analyse SC differentiation dynamics, Smith *et al* illustrated a stochastic spatial model of differentiation: the differentiation decision is slow when the target SCs are surrounded by other SCs, while the differentiation increases by three-fold when surrounding cells are differentiated cells. Similarly, it was found that the self-renewal ability of epidermal SCs can be driven by neighbouring cell differentiation events [30]. Cell adhesions and physical interaction are considered involved in underlying mechanisms, for example, E-cadherin, the key component of adheren junction, affects multiple signalling mechanisms through cell-cell interactions [31]. Besides, ECM adhesion molecules such as integrins not only serve a mechanical function but also acts as signalling molecules which mediate the both maintenance and differentiation of the SCs [32]. Interestingly, independent studies illustrated that integrin promotes differentiation [33] while E-cadherin adhesions maintain pluripotency of the human PSC [34], these two adhesion molecules compete for activation of the Rho-ROCK-myosin II signalling and the crosstalk contributes to the heterogeneous cell fate patterning in SC colony [35]. Another interesting finding is the vital contribution

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of mitochondria towards cell differentiation. Mitochondrial variability emerges from two sources: stochastic mitochondria inheritance during mitosis and function variability. Evidence suggests that high mitochondrial performance can stabilize the undifferentiated state of SC and the mitochondrial noise has the potential to dominate over other extrinsic factors and directly induce the change in regulatory gene transcription rate in SCs [36]. Finally, SC heterogeneity can be caused by environmental epigenetic modifications as well. Exposure to chemicals and stress conditions can induce epigenetic changes and subsequently affect the gene expression [37]. Different types of epigenetic modifications and varied temporal kinetics of epigenetic memory have different effects on the gene expression [38]. Tet protein is a typical example to show how epigenetics can lead to SC variability. Tet proteins catalyse DNA demethylation and are critical for the maintenance of *Nanog* expression in mouse ESCs (mESCs), thereby regulating their fate decision and self-renewal [39].

4. Future direction and perspective

Studying development stochasticity is challenging. One of the most important obstacles would be the technical limitations. Traditional methods for studying cell-cell variability relied on measuring gene expression levels using techniques like reporter gene infusions. However, recent technological advancements have revolutionized the field by introducing accurate and efficient methods for detecting variability at the single-cell level including single-molecule microscopy, single-protein measurement, and single-cell RNA sequencing, enabling the examination of gene expression patterns at unprecedented resolution and accuracy. Moreover, combining gene expression with spatial or temporal measurement allows a better understanding of molecular spatial localization and expression pattern inheritance. The recent study also successfully integrated transcriptome and lineage measurements to create an HSC fate map on a continuous transcriptional landscape for studying cell fate determination and understanding the differentiation mechanism [40]. Future direction will involve optimizing and combining different techniques to explore the correlation between different molecules and ultimately understand their specific roles in complex metabolic and signalling pathways [4]. Another research barrier arises from the interaction between different regulatory factors, making it difficult to separate and identify the influence of each factor. This issue can be addressed by the direct intervention of molecular variability (e.g. in vivo editing by applying small RNA or CRIPSR/Cas9 techniques) or using the "-omics" technique to quantify the relationship between different variables. The next step after identifying the stochasticity at the single-cell level is to identify whether the stochasticity is a cause or consequence of the system, understand its correlation with other factors, and further investigate its functional mechanism. Therefore, functional assays and perturb experiments will be necessary. Lastly, further investigation of the evolutionary theory behind the development of stochasticity will be essential. As discussed earlier, the stochasticity in organismal development can be harmful and natural selection acts to reduce the randomness in dosage-sensitive genes by slowing down the genetic expression variability, however, this randomness is also vital in allowing organisms to adjust and increase survivability. Therefore, studying how the benefits and drawbacks of stochasticity are balanced throughout evolution becomes crucial [41].

5. Conclusion

In conclusion, it is evident that stochasticity not only exists but also plays a significant role in organismal development. Organisms benefit from stochasticity to adapt and survive in changing environments, while stochasticity can also contribute to diseases and ageing processes. Given the decisive role of SCs in organismal growth and survival, they can be regarded as a developmental model system whose heterogeneity and randomness reflect the stochastic nature of general development processes. The heterogeneity aids in balancing the self-renewal and differentiation processes of the whole SC population, providing it with resilience, adaptability, and plasticity. Both intrinsic and extrinsic factors contribute to the cell-cell variation, with TF (e.g. NANOG) heterogeneity being a key intrinsic factor and this random fluctuation of TF expression can be influenced by mechanisms like transcriptional bursting and allelic switching. Additionally, cell cycle variability also affects cell fate determination. SC development can also be influenced by extrinsic factors, notably the stem cell (SC) niche, through

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various mechanisms including secreted factors, cell-cell interactions, and cell-ECM interaction. Mitochondrial variability and environmental epigenetics are two other causative factors contributing to the overall stochasticity. These factors further highlight the complexity and dynamic nature of SC regulation. Although significant progress has been made in studying stochasticity, technical barriers still need to be addressed. Further investigation is crucial to gain a deeper understanding of how stochasticity affects developmental processes and the interplay between determinism and stochasticity throughout development.

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