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Épigénétique et mémoire cellulaire

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ÉPIGÉNÉTIQUE ET MÉMOIRE CELLULAIRE

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La série de cours « Mémoire cellulaire au cours de la vie » est disponible, en audio et vidéo, sur le site internet du Collège de France (https://www.college-de-france.fr/fr/agenda/cours/memoire-cellulaire-au-cours-de-la-vie), ainsi que le colloque du même nom (https://www.college-de-france.fr/fr/agenda/colloque/memoire-cellulaire-au-cours-de-la-vie).

ENSEIGNEMENT

COURS – MÉMOIRE CELLULAIRE AU COURS DE LA VIE

In this series of lectures, I explore the topic of how mammalian cells retain identity, functionality and potential, throughout life. The summaries below are based on the material provided in the support slides for each lecture, where all references for text and citations can be found[1].

Cours 1 – Mémoire cellulaire/*Cell memory* : introduction

1er mars 2021

Cell memory is the process by which all progeny of a parent cell retain the *specialization* of that cell (cell *identity* and/or cell *potential*), after the cue/signal has gone. It is also the process by which a cell maintains its identity and it functionality

1. https://www.college-de-france.fr/fr/agenda/cours/memoire-cellulaire-au-cours-de-la-vie.

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over time (in non-dividing cells). Cell memory also concerns the remembrance of previous exposures to environmental signals or stresses, metabolic interactions, infections (particularly for quiescent adult stem cells and cancer cells). The topic is intimately linked to epigenetics, as defined originally by Waddington, and then later by Riggs, Holliday as the process whereby cells retain differential gene activity states over time (whether in dividing or non-dividing cells), without evoking differences at the DNA sequence level.

Cell memory may also be linked to memory in the brain—but this is not the focus of these lectures.

The cell is the unit of life. Every organism is made up of cells—living cells come from other pre-existing cells as postulated by Virchow: omnis cellula e cellula, 1855. The cells in different tissue/organs (~200 different cell types in total) have to maintain their specialised role over very long periods of time. Most cells in the body are non-dividing (either quiescent or post-mitotic. It is still not clear if a maximum lifespan exists for non-dividing (postmitotic) cells of mammals. On average the cells in a human body are replaced every 7 to 10 years, and this masks a vast variability in lifespan across the different organs of the body: from days to years, to an entire life and even beyond. By transplanting neurons from old mice into brains of longer-lived rats it has been found that these old mouse neurons were still healthy after living for two whole mouse lifespans.

I first present the revolutionary studies, including the Human Cell Atlas project[2] and the NIH Biomolecular Atlas Project, that have been carried out in recent years aiming to better identify and classify the diversity of cell types that are present in different organs, thanks to the power of single cell technologies. These have enabled mapping human cell phenotypes to genotypes mainly using RNA (transcriptomes) as a readout (e.g. see Camp *et al.*, 2019: "Mapping human cell phenotypes to genotypes with single-cell genomics"). Whilst this approach does have some limitations, as the functional molecules in cells are, for the most part proteins, and not RNAs, nevertheless the patterns of gene expression based on RNA so far have clearly identified previously known cell types, new sub-types, new cell states, as well as new cell types altogether. The human body and those of many other organisms are being mapped at cellular resolution which is opening up a whole new era of understanding how organisms develop, function and misfunction in disease, at the cellular and molecular levels. These approaches also allow the variability between individuals and within tissues of the same individual, in different states (age, health etc) to be disovered. Single cell profiling reveals molecular phenotypes that underlie cell identity, but also heterogeneity and cell to cell variation.

Indeed, no two cells in a cellular population are necessarily the same, and no two individuals of a multi-cellular species are identical—not even if they are genetically

2. https://www.humancellatlas.org.

identical e.g. monozygotic twins or clonal organisms. Besides sex, age is the most important non-genetic source of inter-individual variability. Increased transcriptional and epigenetic variability with age occurs in genetically identical twins and unrelated individuals and is also referred to as "epigenetic drift." The epigenetic impact of accumulating environmental exposure, and its interplay with genetic and stochastic factors, provides an explanation for the frequently observed discordance of disease between monozygotic twins and the increase of common diseases with age.

I also explain how in the context of polymorphic individuals, the expression of either or both alleles of some genes in the genome, in contexts such as X chromosome inactivation in females, or random monoallelic expression in some cells, can lead to clonal populations of cells that differ, not just between but also within individuals: in other words, cells with the same genotype can display very different phenotypes, thanks to expression of one or other allelic variant. This underlies the cellular mosaicism that characterises female mammals due to the random inactivation of the paternal or maternal X chromosome.

Today, there are several genomic techniques and computational methods for tracking cellular development and follow cellular memory using barcoding of single cells. These high-throughput single-cell genomic methods enable a global view of cell type diversification via transcriptomes and epigenomes, in particular DNA methylomes which provide an easy albeit costly, method to track cells.

Diving into the epigenetics of cellular memory, I discuss the emergence of the notion that epigenetics is linked to the memory of differential cell fates. Indeed, Waddington's original formulation of epigenetics, implied changes in gene regulation and activity during development; the notion of heritability was not an explicit component. In the 1970's-80's a major shift took place in the use of the word, to include the notion of *transmission* or *heritability* of gene expression states. In particular, observations in cultured cells suggested that there is indeed a memory in cell type that is somatic heritable. In 1975, Robin Holliday and Art Riggs independently postulated that DNA methylation might affect gene expression, and that changes in DNA methylation might explain the switching on & off of genes in development. They predicted existence of enzyme(s) methylating a particular region of DNA—either by sequence-specific binding, or via interaction with other proteins that were sequence specific. DNA methylation patterns could thus be heritable, if maintenance methylases existed that could recognize hemi-methylated DNA soon after replication, but do not act on unmethylated DNA. This was proposed to explain the heritability of the methylated and non-methylated DNA and the heritability of a given pattern of gene activities, and this cell identity.

Insights into how dividing or replicating cells in culture might "remember" their differentiation state with such high fidelity, were subsequently obtained from studies in the 1980's by Peter Jones and others, when cells were treated with a chemical agent that interferes with DNA methylation (5-Aza-C) for several days, whilst they were dividing and found to change their original identity under these conditions, which

they would never normally do. In the decades that followed these visionary experiments, a multitude of epigenetic marks have been identified, that help to ensure that precise gene expression profiles are established and/or sustained, during development and that help to keep the rest of the genome in a repressed state, to prevent accidental activation of genes and repeats. Genetics and biochemistry studies in different organisms, including yeast, Drosophila fruitfly and mice, have established the key role of chromatin in cellular memory of dividing & non-dividing cells as a barrier for aberrant gene expression. Many other factors also play a role including non-coding RNAs, miRNAs and prion-like proteins.

Cell cycle and cell division pose important challenges for the propagation of cell memory: how are states of gene expression or repression maintained through S-phase when the genome become replicated and transcription and chromatin states are disrupted? And how are epigenetic marks duplicated? Can gene activity/repression be maintained through mitosis when the chromatin becomes highly condensed and most transcription is halted?

Recent studies by labs such as Anja Groth, Danny Reinberg and others, using new technologies to label and purify chromatin at different stages of the cell cycle, combined with genetics, have started to dissect the molecular basis for the propagation of functional chromatin states propagated when cells divide. These studies have revealed that different modes of inheritance exist for chromatin that is active (transcribed, euchromatin) or inactive (heterochromatin). It seems that the act of transcription, via transcription initiators, helps to re-establish the open chromatin state after replication. On the other hand, repressed chromatin, (some) repressive marks can be propagated as domains thanks to so-called Reader-Writer systems, whereby the mark laid down by one protein (writer) is detected (read) by another from the same complex.

The mechanisms of cellular memory in dividing cells may be similar or different to non-dividing cells. Cell cycle is central to the establishment and maintenance of cell fates. Cell fate switches are often linked to cell cycle transitions in dividing cells. Terminal differentiation is often associated with exit from the cell cycle. Exit from cell cycle also poses challenges for the long-term maintenance of cell memory. Quiescent adult stem cells exist in a reversible G0 cell cycle state (or in some cases in G2 state), which is distinct from differentiated and senescent cells, that exist in an irreversible G0 cell cycle state. The phenomenon of cellular senescence was discovered in human cell lines that exhaust their replicative potential. It is characterized by cell-cycle arrest in the G1 or possibly G2 phase, which prevents the proliferation of damaged cells. On the other hand, cellular quiescence concerns a reversible growth arrest state secondary to scarce nutrition and growth factors, takes place in the G0 phase. Cellular senescence occurs during embryonic development and can be induced by cellular impairment, including DNA damage, telomere shortening or dysfunction, oncogene activation or loss of tumour suppressor functions, epigenetic changes and organelle damage.

Little is known about how cell identity, transcription, chromatin states, and genome integrity are maintained in non-dividing cells. This is of crucial importance as most cells in an adult are in fact quiescent or non-dividing. This is nicely described by Urban and Cheung (2021) in a review entitled "Stem cell quiescence: The challenging path to activation cellular quiescence is a reversible growth arrest state." However, in response to extracellular environment, some quiescent cells (adult stem cells) are capable of resuming proliferation for tissue homeostasis and tissue regeneration.

The first lecture was concluded with a general overview about adult stem cells (SCs), very much based on a review by Gula and Fuchs (2021). Subpopulations of adult stem cells remain quiescent and reside in their specialized stem cell niches. Within the niche, they interact with a repertoire of niche components. This niche integrates signals that maintain quiescence and also enable stem cells become triggered to divide, differentiated and regenerate a tissue. Importantly, aberrant niche activities perturb stem cell quiescence and activation, compromise stem cell functions, and contribute to tissue aging and disease pathogenesis. It is now recognized that these are key for maintaining tissue homeostasis. They are also responsible for maintaining tissue structure and function by replacing dying cells and balancing proliferation with differentiation.

SCs are usually rare in a niche, which is a complex, specialised microenvironments that control SC lineage outputs depending on localized tissue needs. In their niche, SCs are connected to supporting cells, protected from harmful stimuli, and regulated by appropriate activating signals. SCs respond to environmental perturbations and tissue stressors in order to restore the tissue to homeostasis and to protect it from secondary assaults. Critical to their function are two key processes, SC lineage plasticity and SC memory. Ageing can lead to loss or exhaustion of SCs and one of the big questions is whether this is due to intrinsic or extrinsic factors. During steady-state (homeostasis) some SCs e.g. epidermis, give rise to only one specific cell fate, but others e.g. in the hair follicle (HF), intestine, or hematopoietic system give rise to multiple lineages. Temporally, SC renewal can be continuous (epidermis, intestine, and lung airways), very slow (in muscle and sweat glands), or in bursts of regenerative activity (HFSCs and lactating mammary glands). There are many outstanding questions in the field of adult SCs. These include, how do they replace neighbouring cells after tissue damage? How do they adapt to a local dynamic environment? Do they retain information of previous stressors to better guide cell fate decisions at later times?

In summary this first lecture covered:

- cell identity must be maintained over life—yet cell to cell variation is frequent and increases with age;
- cellular memory may be ensured by many mechanisms, including chromatin and chromosome folding as well as non-nuclear processes;

- chromatin memory is essential to buffer against changes in cell identity/fate, and ensure heterochromatin stability (prevent aberrant gene expression, repeat activity, centromeric instability…);
- repressive chromatin is truly epigenetic, self-templating during S-phase and remaining associated through Mitosis; domains of repressed chromatin may also be required to ensure memory and stability in quiescence?
- active euchromatin is dependent on transcription factors and transcription to be re-established during DNA replication and in G1 although some TFs show mitotic bookmarking;
- chromosome folding is dynamic during the cell cycle and may facilitate gene regulation and provide stability of genome organization through development and in quiescent cells;
- chromatin plasticity is essential during development and in some tissues to respond to hormonal and other signals ⇒ equilibrium vs epigenetic stability ("domains" rather than single nucleosomes are the functional units of chromatin);
- stress-induced changes can impact chromatin states—that are usually reversed but may sometimes lead to heritable changes in the soma and a memory in quiescent stem cells;
- chromatin states are globally erased in the germ line of all organisms. Evolution appears to have gone to great lengths to prevent the carry-over of irrelevant (or deleterious) epigenetic information that would destabilise organisation of the next generation.

Cours 2 – Stabilité et plasticité au cours du développement/ *Stability and Plasticity during Development*

8 mars 2021

In the second lecture, I discuss cellular memory during embryogenesis: the process of establishing cellular memory during development, the epigenetic dynamics during development, the epigenetic machinary that enables cellular memory and the notions of stability and plasticity. I also elaborate on different technologies that can trace cell identity and cell fate during embryogenesis and detect the establishment cellular memory during development.

Decades of research have been dedicated to the study of cell fate changes in development. These led to the generally accepted view that, in vivo, differentiated cells are irreversibly committed to their fate. However, reprogramming of somatic cells, either by transfer into enucleated oocytes pioneered by Briggs and King, as well as John Gurdon in the 1950s, or fusion with other cell partners (work of Helen Blau) or through the ectopic expression of transcription factors (work of Yamanaka), have revealed a remarkable plasticity in differentiated states and the capacity to erase or change epigenetic marks and gene expression patterns.

The way in which cellular memory is progressively established during embryogenesis is presented and the examples where such memory can be partially or globally erased in development are shown, mostly based on studies conducted in the mouse. Single cell genomics as well as imaging approaches have provided unprecedented insights into the changes that occur at different times of development. Using such approaches, previously uncharacterised cell types or functional cell states may be identified by 'clusters' of cells with similar transcriptomes that are distinct from other groups of cells based on scRNA-seq data. There are several multi-omic atlases of mouse embryonic development and specific tissues and lineages, and their trajectories. Techniques that allow cellular memory to be explored, integrate various components of the epigenome into multi-omics measurements. This enables cellular heterogeneity to be identified at different time scales. Measurements range from transcription factor occupancy and initiation of transcription to heritable epigenetic marks such as DNA methylation. Together with techniques in which cell lineage is recorded, this multilayered information provides insights into a cell's past history and its future potential. Cell lineage can be traced by CRISPR scarring approaches in which each cell and its descendants within a lineage are linked by unique mutations or barcodes. DNA modifications (e.g. methylation) can also be used to track lineage based on their inheritance and on errors in their maintenance at DNA replication.

Cours 3 – Maintien de l'identité cellulaire dans les cellules non prolifératives

15 mars 2021

In the third lecture, I discuss in more molecular detail how cell identity and cellular memory are established and maintained. I first discuss the early decisions that direct cell fate in mammalian embryos. Initial heterogeneity in expression of some genes, seen at the 4-cell stage mouse embryo, appears to be partly dependent on chromatin remodelling complexes. This is followed by progressive restriction of cellular plasticity from 4-cell stage. Positional cues start to play a role at ~8-16 cell morula stage, with inner cells tending to form inner cell mass (which will lead to the epiblast = soma + germ line; primitive endoderm) and outer cells tending to form trophectoderm (TE – extra-embryonic tissues). Key transcription factors are known to be essential to determine cell fate and establish cell lineages of the early embryo. Chromatin factors also contribute by providing permissive (or non-permissive) environments for cell fate, and/or predispose a cell towards a particular lineage. Chromatin marks and DNA methylation also progressively lock in active and inactive states of gene activity. Years of work by multiple labs, using conditional knock out strategies where a mutation can be induced at different stages of development, have shown that chromatin modifiers can have one or more roles in development.

As an example, to illustrate this I focus on one specific chromatin factor, the histone methyltransferase G9a, that is responsible for establishing one form of histone H3K9 methylation (H3K9me2). The G9a knock out is lethal at ~E9.5. In vitro H3K9me2

domains extend into developmentally regulated genes. G9a targets de novo DNA methylation and H3K9me2 inhibits reprogramming of somatic cells. The work of Jan Zylicz in Azim SUrani's lab showed that G9a has at least two roles during development. Maternal G9a pre-sets lineage segregation, similarly to several other maternally-contributed enzymes that pre-set the embryo for later development. Repressive marks accumulate globally but silence specific transcriptional programmes. G9a-mediated spreading of H3K9me2 allows for rapid inactivation of the regulatory elements (enhancers) of some genes that are critical to establish the germ line programme.

The notion of erasure of cellular memory and re-establishment of new cell identities is crucial in early development. At fertilization the highly specialized states of the sperm and egg cells must be "forgotten"—the epigenomes reprogrammed—and a new state of totipotency (with the potential to form all cell types in an organism) must be established in the zygote. Such global reprogramming is essential for embryogenesis to successfully produce the next generation (sources include reviews such as Xia and Xie, 2020). However, some parts of the parental (maternal and paternal) genomes carry over an epigenetic memory from the gametes. This has been called genomic imprinting and was thought to be limited to a hundred or so genes across the genome (this has been extensively covered in my lectures of previous years). Recent work from several laboratories (e.g. Du *et al.*, 2017; Ke *et al.*, 2017; Du *et al.*, 2020), including my own together with the laboratory of Peter Fraser (Collombet *et al.*, 2020), have studied early patterns of gene expression and chromatin states in pre-implantation embryos. We have revealed that there is early, potentially transient, monoallelic expression of many more genes than previously thought. The gene expression, as well as the accompanying 3D chromosome architecture and chromatin states suggest that there is indeed a parental memory carried over from gametes to embryos. By performing allele-specific analysis of chromosome architecture during early mouse development, from the fertilized egg to the blastocyst and report maternal and paternal specific folding in a minority of the genome and this corresponds in many cases with allele-specific expression. The X chromosome was of particular interest, given that the paternal X is specifically repressed during this period of early development due to a transient maternal imprint that represses Xist, the key regulator of XCI which entails Polycomb marking as shown by the lab of Yi Zhang (see Loda *et al.*, 2022 for review). The Collombet *et al.* study reveals how 3D topology predicts the region that defines the imprint on the maternal X that prevents it from being silenced (upstream of the Xist locus), and also reveals changes in 3D structure as X chromosome-wide silencing occurs. The maternal Xist imprint is essential for normal development, and we show that the deletion of the region responsible for the maternal imprint leads to mis-regulation of Xist. This type of transient cellular memory for specific loci, may be essential for early steps of development in some cases such as Xist, but is then lost at later stages. The exact reasons why this type of transient, parental, cellular memory might have evolved are still unclear. Furthermore, very few studies have explored the question of how such transient parental imprints influence gene expression patterns during development.

An overview of the epigenetic changes during X-inactivation throughout development is given to illustrate both the technologies used to distinguish alleles and follow gene activity at the single cell level, as well as the lessons learned about the rapid initiation and then reversal of repressed gene activity, with full reactivation of the inactive X chromosome, at specific stages of early development. The work from my lab and others has demonstrated how epigenetic reprogramming is linked to establishment of pluripotency, and how epigenetic changes are also essential for the exit from pluripotency and differentiation. Furthermore, work from my lab in the context of the X chromosome, on the potential role of genes expressed from both the active and inactive X chromosomes (XCI escapees) in XX females is discussed. This demonstrates how the silent memory state of the inactive X chromosome, may need to be very specifically overridden for some genes, presumably to ensure female-specific functions.

In the last part of the lecture, I discuss the evidence for cell memory states that are set up early on in development, in order to ensure appropriate gene activity only at later stages or upon stimulus. Our knowledge about establishing the memory of somatic cell lineages through pioneer and bookmarking transcription factors as well as chromatin states is discussed. The importance of repressing and activating, or priming genes, as lineages are established is also presented. Several examples are given. The role of key transcriptional regulators: Oct4, Sox2, and Nanog in ensuring two different pluripotency states (naïve or primed) is covered, as an example of how cells are primed for lineage specification by rewiring gene regulatory networks via specific TFs using alternative enhancers and partners. In another example, during liver development, master transcription factors bind to their DNA targets, either in a temporally stable or in a dynamic manner. This study demonstrates that early and persistent binding is necessary, but not sufficient, for gene activation. Further, stable gene expression patterns appear to be the result of combinatorial activity of multiple transcription factors, which mark regulatory regions, a long time before activation. When bound these factors promote progressive broadening of active chromatin domains. Both temporally stable and dynamic, short-lived binding events thus contribute to the developmental maturation of active promoter configurations (Karagianni *et al.*, 2020).

In another example, by Stroud *et al.*, 1017, the deposition of methylated CA (mCA) marks by the DNA methyltransferase DNMT3A is shown to occur within specific brain genes during early post-natal life but is potentially important for their regulation throughout later life. The mCA that is recruited during early life, is proposed to fine tune gene expression in the adult brain. This mCA marking is proposed to contribute to the fine-tuning of genes, including those with critical neuronal functions, in a neuronal subtype-specific manner at least in part by differentially recruiting MECP2 to neuronal gene bodies.

Another example of cellular memory that enables genes to be poised for action, concerns Polycomb (Pc) associated marks—another classic epigenetic memory

system. Kitazawa *et al.* (in *Nature Genetics*, 2021), show that, during development, cells are exposed to a variety of distinct environmental signals to which they may need to rapidly respond in a spatiotemporally regulated manner, in order to keep their differentiation schedule. In some cases (particularly in the brain) response to these environmental signals requires rapid, stimulus-dependent, transcriptional responses through induction of what are called Immediate Early Genes (IEGs), encoding TFs—which in turn will regulate activation of specific Late Response Genes (LRGs), thereby driving cell-type-specific differentiation schedules. Before induction, IEGs share key regulatory properties, which poise them for rapid stimulus dependent activation. One question has been to understand how spatiotemporal regulation and specificity of the IEG transcriptional response is achieved in developing cells and also how the untimely induction of IEGs in response to spurious signals can be prevented. A unique (H3K27ac/H3K27me3) bipartite chromatin signature, modulates the rapidity and amplitude of the transcriptional response of inducible IEGs to distinct stimuli during development. Polycomb marks the body of IEG genes and may act as a buffer against untimely high-level expression. Strong stimuli allow for the rapid removal of Pc marking of gene bodies and fast transcriptional induction (active removal, not passive). The bipartite signature regulates the rapidity and amplitude of transcriptional response to stimuli: inhibition of UTX (Kdm6a) and Jmjd3 (Kdm6b) prevents Pc removal and rapid activation. Thus, Pc marking of gene bodies of bipartite stimulus-response genes may establish a threshold to prevent rapid transcriptional induction of IEGs in response to suboptimal and/or nonphysiologically relevant levels of environmental stimuli. The critical importance of chromatin remodelers in enabling such primed states to be setup during precise developmental windows is touched on and will be the focus of future lecture series.

Finally, the role of 3D chromatin organisation in the poised or pre-marked state is also discussed, as studies suggest that long range interactions may also be involved (Boyev *et al.*, 2018). This is a topic of active exploration.

Cours 4 – Stabilité génétique et épigénétique au cours du vieillissement

22 mars 2021

The topic of this lecture mainly concerns cell memory in non-dividing cells and quiescent stem cells and discusses epigenomic and protein stability in different cell types with age. The various hallmarks of quiescence are discussed, as is the topic of adult stem cells, their discovery, their identities and their roles in tissue homeostasis over life.

Approaches to detecting the age of cells and proteins in adults are first presented. Adult tissues contain quiescent stem cells and post-mitotic cells as old as the organism. Proteins and organelles must be protected or renewed to maintain cell homeostasis,

but the actual ages of cells and proteins have only recently been properly estimated thanks to the use of high-resolution isotope imaging. In particular the work of Martin Hetzer's laboratory and others have made major contributions in this area (see for example Arrojo e Drigo *et al.*, 2019 and Truscott *et al.*, 2016). This has revealed that adult mouse organs are mosaics of cells of different ages. Neurons in the central nervous system, pancreatic alpha beta and delta cells, and even the liver which has relatively high turnover, contain cells as old as the animal. Cilia have differentially aged structural protein components, with some lasting the lifetime of the organism. In post-mitotic (non-dividing) cells, the only organelle that does not turn over is the nucleus. This organelle contains the genome and the machinery that must ensure its appropriate function and expression. Different strategies seen for nuclear pore maintenance in irreversible and reversible non-dividing cells. By monitoring the replacement of specific, long-lived components of nuclear pore complexes, and nucleosomes (DNA+Histones) during aging in postmitotic cells, it can be seen that nuclear pore complex (NPC) proteins are maintained via a piecemeal process of replacement. Using genome-wide mapping of long-lived histones also revealed specific enrichment of long-lived variants at silent gene loci. There is thus age mosaicism at the level of chromatin organisation across the genome. This obviously has important implications for the memory of appropriate gene expression patterns as chromatin plays a vital role in ensuring repressed or active states of transcription.

The state of cellular quiescence, as quotes from reviews by Elaine Fuchs, Fiona Watt and others, is a dormant but reversible state in which cell-cycle entry and proliferation are prevented. This state of low proliferative activity, experimentally defined by the ability to retain DNA or chromatin labels, is taken as a defining characteristic for adult stem cells (SCs). Stem cells are hypothesized to be slow-cycling to conserve their proliferative potential and to minimize DNA errors during replication. In the 1980s, investigators (Mackenzie and Bickenbach, 1985) howed that a single pulse of tritiated thymidine does not label presumed stem cells in the epidermis and oral epithelium. Rather, their labeling required prolonged administration of the DNA label. Cells that cycle slowly (the presumed stem cells) retain the isotope for an extended period of time and were termed label-retaining cells (LRCs). Unlike most adult stem cells, hematopoietic stem cells are regularly recruited into the cycle, such that 99% of LT-HSCs divide on average every two months, even though the majority of long-term HSCs (LTHSCs) rest in G0 at any given time. Due to the difficulty of identifying the G0 phase of the cell cycle, quiescent cells had remained poorly characterized. However, the use of fluorescent markers fused to specific cell cycle proteins has meant that researchers can now identify and isolate quiescent cells and effectively visualize the G0 to G1 transition (e.g. Oki *et al.*, 2013 from the Kimura lab).

In fact, the term stem cell was coined at the end of the nineteenth century by Boveri and Haecker to propose the notion of a common progenitor cell for distinct blood lineages. The existence of this progenitor, called a haematopoietic stem cell

(HSC), was ultimately proven by McCulloch and Till in the 1960s when mouse bone marrow cells were injected into irradiated mice. They hypothesized that each lump (colony) observed in the spleens of these mice was a clone arising from a single marrow cell (stem cell). Siminovitch found colony-forming cells were capable of self-renewal, which is a key defining property of stem cells that Till and McCulloch had theorized. The discovery of HSCs led to the defining concept of a stem cell as a self-renewing cell positioned at the top of a hierarchy, giving rise to a range of fully differentiated, specialized cell types at the end of the hierarchy's branches. However, it is increasingly clear that the HSC, which is the only cell that can act as a stem cell in the haematopoietic hierarchy, which is strictly unidirectional, may be the exception rather than the rule, as is nicely discussed in a review by Watt and Clevers, 2018.

Adult stem cells in most tissues are rare, difficult to detect, and yet they are essential for tissue homeostasis and their loss or exhaustion over life, clearly contributes to the features of an ageing individual. SCs respond to environmental perturbations and tissue stressors in order to restore the tissue to homeostasis and to protect it from secondary assaults. Two key processes that are critical to SC function are lineage plasticity and memory. Different SCs display different modes of outcome upon environmental stimulus or stress. During steady-state (homeostasis) some SCs e.g. epidermis, give rise to only one specific cell fate, but others e.g. in the hair follicle (HF), intestine, or hematopoietic system, give rise to multiple lineages. Furthermore, temporally SC renewal can either be continuous (epidermis, intestine, and lung airways), very slow (in muscle and sweat glands), or occur only in bursts of regenerative activity (HFSCs and lactating mammary glands).

There is no universal gene marker for adult SCs and only very few cases so far, where a specific gene expression signature is really known. As described by Etzrodt *et al.*, 2014, performing live-cell imaging with sufficient spatial and temporal resolution of adult SCs in mammals poses major technical challenges and remains restricted to extremely few specialized cases. An example is given of live imaging of neurogenesis in the adult mouse hippocampus (Pilz *et al.*, 2018; Bottes *et al.*, 2021). Using intravital imaging (2 photons), neural stem cells and their progeny were visualised over several months. A population of Gli1-targeted NSCs was identified showing long-term self-renewal in the adult hippocampus that contribute to the generation of new neurons in the adult hippocampus. Another population, of Ascl1-targeted NSCs, once activated, showed very limited proliferative activity before they become exhausted. Importantly, single cell RNA sequencing has shown that Gli1- and Ascl1-targeted cells have highly similar yet distinct transcriptional profiles, supporting the existence of heterogeneous NSC populations with diverse behavioural properties. In another example, this time looking at skin, where the basal layer keratinocytes and their organization into distinct long-lived stem cell and transient progenitor populations has been the subject of intense research. Live imaging combined with the use of inducible and light-modulated fluorescent reporters enabled live-cell tracking of individual basal keratinocytes over multiple generations.

This revealed that the fates of epidermal daughter cells are not predetermined, and they appear to have lifetimes that are coupled.

The lessons from the hematopoietic stem cell lineage in terms cellular memory are also discussed, including from a recent study by Cheung *et al.* performing single cell chromatin modification profiling from individuals of different ages and in particular at monozygotic or dizygotic twin pairs. Ageing is associated with reduced hematopoietic output and defective immune cell functions. Differential analysis between younger and older adults shows that aging is associated with increased heterogeneity between individuals and elevated cell-to-cell variability in chromatin modifications. At the level of the epigenomes, most chromatin modifications increase in older subjects for most cell types, except in central memory CD8+ cells. Cheung et al. also show that ageing-related alterations are largely driven by non-heritable factors (both MZ and DZ twins show similar variation to random individuals). The investigation of CD8+ T cells is the topic of another study, by Pace *et al.*, 2018. Unlike naïve and effector T cells, memory cells have unique "stemness" characterstics that allow their long term survival and plasticity to replenish effector pools after renewed antigen challenges. Pace et al. explored the basis of this T-cell memory that can protect against pathogens and cancer throughout life. They demonstrate a role for the epigenetic histone methyltransferase, Suv39h1 in the control of stem cell related memory genes, to establish an epigenetic barrier on the stem/memory gene expression program, and prevent effector cell reprograming into memory cells.

Finally, adult SC hierarchies are discussed with detailed description of the intestinal stem cell niche, as well as those of the liver, the brain and the skin. A large body of work in these different systems, using powerful imaging and tracing approaches, with genetic engineering to target (disrupt or express) genes in specific stages or cell types, as well as single cell genomics, from the labs of people like Hans Clevers. Fiona Watt, Elaine Fuchs, Cedric Blainpain and others, has led to the overarching conclusion that stem cell function in solid tissues may be executed in a diffuse fashion by much larger populations of undifferentiated cells. As described (Watt and Clevers, 2018), in rare cases, this may involve HSC-like, hardwired, professional stem cells. In many cases, SC function is likely ensured by facultative stem cells, which are proliferative, undifferentiated cells that are opportunistically recruited from committed cells, or even from fully differentiated cellular compartments upon tissue damage such as in the liver.

In fact a "stem cell" is one of the most disputed terms in science (Watt and Clevers, 2018). The classic definition of stem cells is that they are at the origin of a lineage, and are self-renewing and multipotent, generating all cell types of a given tissue. Indeed, in the blood, skin and gut, stem cells are the seeds that sustain tissue homeostasis and regeneration. In other tissues like the muscle, liver, kidney and lung, stem or progenitor cells play facultative roles in tissue repair and response to injury. This has led to the conclusion that the modern definition of a stem cell must be functional.

Cours 5 – Perte d’identité cellulaire au cours de la reprogrammation et dans des pathologies

29 mars 2021

In this final lecture, the topic of stem cells and their role in tissue homeostasis is covered, particularly the nature and role of the stem cell niche, and also of stem cell memory. How the niche and/or stem cells change with age, is directly important for how different tissues age at the cellular and molecular levels. I also touch on changes or loss of cellular identity during reprogramming in disease.

The regulation of stem cells that maintain and regenerate postnatal tissues depends on intrinsic events and extrinsic signals originating from their microenvironment or stem cell niche. Stem cells integrate complex regulatory signals from the tissue and systemic factors (circulating) through the niche (Gola and Fuchs, 2021). There are many open questions that are only now being explored as described in a review by Urban *et al.*, 2019: How do SCs change when they become activated? How do SCs replace neighbouring cells after tissue damage? How do they adapt to a local dynamic environment? Do SCs retain information of previous stressors to better guide cell fate decisions at later times? It is clear that lineage plasticity is in part context dependent and that SC fate is finely regulated by local environmental cues.

These concepts and questions are explored with specific examples. The first example, as described in a review by Mashinchian *et al.* (2018), “The muscle stem cell niche in health and disease”, concerns skeletal muscle stems cells. Skeletal muscle is the most abundant tissue of the human body. Its contractile properties are essential for vital functions such as locomotion, postural support, and breathing. It also has important endocrine and paracrine functions, and regulates thermogenesis and systemic metabolism. The stem cell niche in skeletal muscle tissue is a paradigm for a structurally and functionally relatively static niche that maintains stem cell quiescence during tissue homeostasis. Healthy muscle fibers are large and long-lived, non-proliferative syncytia. Satellite cells are closely apposed to these fibers as small, nondividing cells that consist of barely more than a nucleus. Satellite cells can lie dormant for years only to become acutely active upon muscle damage. Upon injury the niche becomes highly dynamic and regenerative subject to extensive structural remodeling and different support cell populations. The stem cell niche that maintains MuSCs in their quiescent state in the absence of muscle injury is composed of two major compartments: the interface with the muscle fibers and the basement membrane.

In the immediate phase following injury, the niche contains debris of degenerated muscle fibers and a high abundance of proinflammatory immune cells. Subsequently, the niche changes into a milieu that promotes the proliferation of MuSCs and that is characterized by extensive ECM synthesis by fibroblastic cells and angiogenesis. In the differentiative phase, anti-inflammatory macrophage subsets become dominant and MuSC-derived myoblasts fuse into young muscle fibers that are reinnervated, and basement membranes mature.

In studies that explore how muscle stem cells can be maintained through symmetric cell divisions (SCDs) and asymmetric cell divisions (ACDs), by the lab of Shahragim Tajbakhsh, it was shown that MuSCs go from symmetric division during early steps of regeneration, to both symmetric and asymmetric division during later steps. Clonogenic cell tracing methods have been used follow the possible asymmetric distribution of transcription factors along with old and new DNA in mouse muscle stem cells during skeletal muscle regeneration. This has shown that transcription factors rather than chromatin (histones) appear to be asymmetrically inherited during initial asymmetric divisions.

The notion of memory of in adult SCs is discussed, in particular exploring how much adult stem cell history can influences stem cell behavior in the context of tissue formation and responses to external stimuli. In the hematopoietic system, the concept of a "trained immunity" has been coined to cover antigen-non-specific, cross-protective, and long-lived innate immune responses, that are proposed to involve epigenetic changes and metabolic reprogramming (see Netea *et al.*, 2020). An increasing body of evidence suggests that trained immunity plays a critical role in humans. This is based on epidemiological data suggesting that live vaccines such as the BCG vaccine, measles vaccine, smallpox vaccine and oral polio vaccine have beneficial, non-specific protective effects against infections other than the target diseases. Studies in HSCs show that they can be educated to imprint mononuclear phagocytes to maintain their memory-like protective capacity against a virulent bacterial pathogen (BCG). Specifically, the initial presence of BCG in the bone marrow (BM) is required for the priming of HSCs, whereas upon subcutaneous vaccination, BCG had no access to BM and thus, no effects on HSCs. In addition, the protective capacity of educated HSCs has been shown to be sustainable in the absence of continued exposure to BCG or memory T cells. Although the cytokine and transcription factor networks in HSCs that drive lineage commitment are known, the regulatory mechanisms that are triggered by BCG to generate educated HSCs are still unknown.

In the last part of this lecture, the theories behind aging, in particular in the context of stems cell memory are discussed. A multitude of theories have been proposed to explain the molecular mechanisms of aging (Medvedev, 1990; Kirkwood, 2005). Processes that involve damage to macromolecules are clearly of fundamental importance in aging theories. These include the somatic mutation theory, telomere loss theory, mitochondrial theory, the altered proteins theory, the waste accumulation theory and network theories of aging. At the cellular level, damaged molecules drive the underlying age-related deterioration in cell function. Increase rates of senescence are observed with aging. Damaged cells are likely to coexist alongside relatively undamaged cells. The question is, what frequency of seriously damaged cells is required to produce significant impairment of tissue function.

At the level of the genome and its functionality, Craig Venter has stated that "it is tempting to speculate that the dynamic process of decay of the somatic genome may

be a stronger predictor of aging than hard-coded features of the germline genome". However, Zhang *et al.* in "The ageing epigenome and its rejuvenation," postulate that the progressive accumulation of ageing-associated epigenetic changes could be a more important driver, leading to aberrant gene expression regulation, metabolic instability, stem cell senescence and/or exhaustion and tissue homeostasis imbalance, all of which contribute to ageing. Indeed, they go on to state that:

> Although the accumulation of nuclear and mitochondrial DNA mutations has clearly been correlated with aging and increasing the burden of mitochondrial DNA mutations can shorten life span, there is no direct evidence that DNA mutations are the proximal cause of cellular aging. Specifically, no experiment has demonstrated that a reduction in DNA mutations leads to an extension of life span. As such, there is currently much interest in the role of epigenetic processes as mediators of the aging process.

Nevertheless, clearly accumulation of somatic mutations can lead to disease over time as illustrated in the hematopoietic system.

Both reduced diversity and increased clonality are found over time in hematopoeitic stem cells (HSCs) and skin stem cells. In a comprehensive review by Ermolaeval *et al.* (2018), the progressive appearance of clones carrying mutations and epimutations (epigenetic drift) has important implications for disease onset with aging. Somatic mutations or epimutations that arise rarely confer a selective growth advantage to the cell in which it occurs. Rather, the cell and its progeny ("clone,") progressively expand over time. Large-scale genetic studies have revealed the prevalence and clinical associations of somatic, clonal mutations in blood cells of individuals without hematologic malignancies. The initiating mutation may progress to cancer if additional cooperating mutations are acquired. "Clonal hematopoiesis" may result if the mutated clone contributes to the production of a substantial proportion of mature blood cells. Importantly, mutations in genes involved in epigenetic regulation (DNMT3A, TET2, ASXL1) account for the majority of mutation-driven clonal hematopoiesis in humans. These mutations are rare in the young but highly prevalent in the elderly, with between 10% and 20% of those older than age 70 harboring a clone of appreciable size. In summary, age-related changes in hematopoietic stem cell (HSC) epigenetic and metabolic state and signals from the dysregulated aging bone marrow (BM) microenvironment can lead to clonal expansion and predisposition to leukemic transformation. The above information was gleaned from Ermolaeval *et al*., 2018 and references therein.

Turning to aging in other tissues, recent single cell approaches have established the aging cell RNA atlas in ageing mouse (The Tabula Muris Consortium, 2020). Changes in gene expression with age were due to both changes in the numbers of cells in a population and changes in the gene expression levels in each cell. Tissue composition changes with age, for example proportion of hepatocytes decrease in the liver, being replaced by immune cells and pro-inflammatory cells. In the bladder, the mesenchymal compartment decreased by a factor of three and urothelial

compartment increased by similar amount. Furthermore, there is increased clonality of both B- and T-cell repertoires with age. This may explain why older individuals have a higher vulnerability to new infections and lower benefits from vaccination compared with younger individuals. The most significant changes in diversity with ageing was seen for immune cells that originate from the brain and the kidney. For example an expansion of a pro-inflammatory subset of microglia in the ageing brain, which may have implications for diseases such as Alzheimer's. Finally, full-length transcript data allows an analysis of the accumulation of somatic mutations with age (SNPs). Tongue and bladder cells were found to be the most affected.

In skin epidermis, the identity of old dermal fibroblasts becomes undefined and noisy and they start to acquire adipogenic traits (Salzer *et al.*, 2018). This loss of cell identity may be a possible mechanism underlying aging. A single cell transcriptomic atlas of the aging human epidermis using human skin from donors of different ages, revealed cell-type-specific aging-associated down-regulation of growth-controlling transcription factors including HES1 in fibroblasts and KLF6 in basal cells. Fibroblasts have the highest level of aging-related transcriptional variability amongst the eleven different cell types identified in the skin (Zou *et al.*, 2021). In a study by Enge *et al.*, 2021, single cell transcriptomic analysis of the human pancreas has identified aging signatures of gene expression and somatic mutation. Understanding the mechanisms that underlie the generation and regeneration of β cells in the pancrease is crucial for diabetes. Single-cell technologies applied to β cell differentiation uncover intermediate cell states, cellular heterogeneity and molecular trajectories of cell fate specification. Age-dependent transcriptional noise and compromised Langerhans islet identity are detected and individual endocrine cells in aging people were found to be more likely to express irrelevant hormone genes such as glucagon expression in β cells or insulin expressed in α cells. Thus it seams that there is ncreasing "confusion" in cell identity with age. A model for β -cell failure in Type 2 diabetes is that this is indeed due to loss of β -cell identity, due to metabolic stress.

There is also a clear role of systemic factors and changes in the local microenvironment affect stem cell function during ageing (see Ermolaeval *et al.*, 2018). The environment of most stem cells is in a specialized microenvironment or niche, the property and nature of which vary depending on tissue. The niche provides specific cues such as differentiation and self-renewal-regulating signals, adhesion molecules, spatial organization and metabolic support to stem cells. Thus the niche is essential for the control and regulations of basic functions of stem cells and in protecting them from the accumulation of molecular damage and toxins, for example, microorganism-derived factors in the intestine. The intimate relationship between stem cells and their niche can be influenced by multiple factors, including ageing, diet and systemically acting factors, which include metabolites from the host microbiome.

In the brain the ability of NSCs to proliferate and give rise to new neurons decreases dramatically with age (Pnegredo *et al*, 2020). *In vivo* labeling and

microscopy has revealed a decline in neurogenesis in both sub-ventricular zone (SVZ) and hippocampal neurogenic niches during aging, with increased NSC dormancy, decreased NSC self-renewal, a decline in neuronal fate commitment, and increased NSC death. Both intrinsic molecular changes of SVZ NSPCs during aging, and alterations of extrinsic molecular dignals in the SVZ niche are associated with its age-related neurogenic decline.

In the muscle, aging is characterized by a progressive decline of physiological integrity, leading to the loss of tissue function and vulnerability to disease. Skeletal muscle has an outstanding regenerative capacity that relies on its resident stem cells (satellite cells). Skeletal muscle regenerative capacity declines with aging. Muscle stem cell number and function decline with aging. It is believed that MuSC aging is also caused by both extrinsic and intrinsic alterations. Importantly, old muscle stem cells can be rejuvenated by youthful environmental factors and interference with age-associated intrinsic changes can rejuvenate stem cells.

As discussed by Zhang *et al.* (2020) in Nature Reviews Cell and Molecular Biology, in some recent studies, aged cells from centenarians or patients with progeroid syndrome, have been successfully used to generate human induced pluripotent stem cells (iPSCs), although with lower efficiencies than young fibroblasts. Transcriptomes of fibroblasts differentiated from iPSCs derived from centenarians were similar to those of fibroblasts derived from human embryonic stem cells, indicating successful rejuvenation of the transcriptome. Progeroid-derived iPSCs were able to give rise to MSCs without an apparent decline in differentiation efficiency, however these MSCs exhibited accelerated senescence and re-established an 'aged epigenome' after extended in vitro culture. These studies suggest that the ageing epigenome can be reset to a younger state after reprogramming to pluripotency. This can be referred to as a senescence reversal process, despite the fact that the derived iPSCs carry the disease-causing mutations in some instances.

Studies in the mouse have shown that in vivo induction of reprogramming factors triggers two divergent cellular outcomes, cellular reprogramming and damage-induced cellular senescence. Senescence-associated secretory phenotype (SASP), particularly IL-6, promotes in vivo reprogramming, suggesting that SASP enhances cellular plasticity in tissues. This has important implications for rejuvenation, tissue regenaration and cancer development in multicellular organisms. Furthermore, beneficial paracrine effects of tissue-injury-induced SASP have been found on reprogramming in vivo.

In conclusion to this final lecture, it is clear that single-cell analyses, lineage tracing approaches and live cell imaging represent technological revolutions that have enabled substantial evolution in the notions of cell identity, cell fate and cell plasticity. They have also provided important insights into the cell and molecular changes that happen over time and during aging in particular, and the impact this can have in tissue homeostasis.

These lectures have explored the notions of cellular memory both in dividing and non-dividing cells, during development and also in the context of adult stem cells which come in multiple flavours and are best defined by function rather than phenotype. The mechanisms orchestrating development (epigenesis) and those propagating cell identity across the cell cycle have been discussed. The questions discussed include how cell function can be maintained over time, and the important role of long-lived somatic cells and proteins to ensure this, and also how cell identity is maintained over time, in the face of environmental and stochastic events. The importance of events both in stem cells and in their niche during aging are being revealed with the discovery that molecular damage and increased cell to cell variation and that stem cell decline in function can be reversed in some cases by reprogramming and resetting epigenomes.

COLLOQUE – MÉMOIRE CELLULAIRE AU COURS DE LA VIE

Organisé par Edith Heard au Collège de France, le 14 juin 2021.

Programme

- Hans Clever : « Organoids to model human diseases » ;
- Shahragim Tajbakhsh : « Recalling skeletal muscle properties in time and space » ;
- Laia Richart-Gines : « Loss of Xist impairs human mammary stem cell differentiation » ;
- Silvia Fre : « Lineage commitment and plasticity of mammary stem cells » ;
- Alain Prochiantz : « Non-cell autonomous OTX2 transcription factor mediates cerebral cortex adaptation to its early postnatal environment » ;
- Leila Perié : « Hematopoietic stem cells: Clonal evolution in blood cancer » ;
- Allison Bardin : « Insight into stem cell lineage decisions through chromatin state transition modelling » ;
- Luigia Pace : « Epigenetic and transcriptional control during T cell fate commitment » ;
- Fiona Watt : « Exploring cellular heterogeneity in human skin ».

RECHERCHE

The Heard group focuses on epigenetic processes such as X-chromosome inactivation, in order to learn more about the basic principles of gene regulation, and to explore the roles of chromatin modifications, chromosome organisation and non-coding RNAs on gene expression in development and disease. It is currently based at the EMBL and an outline of the team and our activities can be found here: https://www.embl.org/groups/heard/.

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