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Interplay between the nucleus and the microtubules : role in the regulation of chromatin organization in hematopoietic stem cells

Stéphane Biedzinski

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Stéphane BIEDZINSKI

**Interplay between the nucleus and the
microtubules: role in the regulation of chromatin
organization in hematopoietic stem cells**

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in the CYTOMORPHOLAB

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Interplay between the nucleus and the
microtubules: role in the regulation of chromatin
organization in hematopoietic stem cells

Rôle de l'interaction entre le noyau et les
microtubules dans la régulation de l'organisation de
la chromatine dans les cellules souches
hématopoïétiques

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Résumé substantiel

Les cellules souches sont caractérisées par leur capacité d'auto-renouveaulement et de différenciation. Autrement dit elles sont capables de à maintenir et de changer leur identité, afin de conserver au cours du temps le nombre de cellules des différentes populations cellulaires d'un organe. Ces cellules sont centrales dans l'homéostasie d'un organisme.

Historiquement le contrôle de l'identité cellulaire a été étudié du point de vue biochimique. En effet, certaines molécules sont capables de lier des récepteurs à la surface des cellules et d'activer ce que l'on appelle des voies de signalisations. Ces voies sont formées par des activations en chaîne de protéines (cascade d'activation) qui ont pour finalité la translocation dans le noyau d'une protéine capable d'activer (ou inhiber) un facteur de transcription, lui-même capable de modifier l'expression de gènes. Ces voies de signalisations vont se combiner en un réseau complexe capable de déterminer l'identité cellulaire.

S'ajoutant à ces voies biochimiques, de plus en plus d'études soulignent l'importance des signaux mécaniques dans la régulation de l'expression génique: c'est la mécanotransduction. Ces mécanismes passent principalement par l'intermédiaire du cytosquelette et surtout le cytosquelette d'actine qui est étroitement lié à la forme de la cellule. Par exemple, en fonction des conditions d'adhérence, une cellule est étalée ou au contraire confinée et cela a des conséquences sur l'organisation de l'actine. Ces changements d'organisation vont engendrer l'activation (ou l'inhibition) de facteurs de transcription. De cette manière la cellule est capable de sentir son environnement physique et de s'y adapter.

Comment est alors définie l'identité cellulaire ? L'ADN, support de l'information génétique, situé dans le noyau, interagit avec un ensemble de protéines et forment une structure: la chromatine. En modifiant chimiquement l'ADN lui-même ou ces protéines il est possible de moduler la structure de la chromatine. Les principales protéines impliquées dans la formation de la chromatine sont les histones. Ces protéines servent de support sur lequel l'ADN s'enroule, et de fait, en fonction de cette enroulement régulé par des modifications chimiques, l'ADN est plus ou moins compacté (hétérochromatine contre euchromatine). Cette compaction limite l'accès aux machineries de transcription qui empêchera donc l'expression de gènes. Toutes ces modifications chimiques portées par la chromatine sont regroupées sous le terme de marqueurs épigénétiques.

La chromatine est précisément organisée dans le noyau en fonction de son état de condensation. Les régions actives (non condensées) se regroupent entre elles, de même que les régions inactives (condensées) se regroupent entre elles. Généralement les régions inactives se retrouvent positionnées en périphérie, ancrées à l'enveloppe nucléaire par un réseau de protéines: la lamina. Dans ce cas, les forces appliquées au noyau sont donc transmises à la chromatine et peuvent donc potentiellement impacter son organisation. En effet, il a été montré que des déformations de l'enveloppe peuvent influencer la conformation des protéines de la lamina, la compaction de la chromatine et son état de transcription. Les propriétés mécaniques du noyau, conférées par la lamina, sont également des facteurs à prendre en compte dans la transmission de forces à la chromatine. Par exemple, plus l'enveloppe est rigide plus il sera difficile de déformer le noyau et donc d'avoir un impact sur l'organisation de la chromatine. Tous ces éléments illustrent l'importance de la lamina dans la mécano-transduction ainsi que les modalités du contrôle mécanique d'expression des gènes.

Comme mentionné précédemment, le cytosquelette est l'élément principal générant des forces sur le noyau. Plusieurs études soulignent l'importance du cytosquelette d'actine dans ce phénomène, particulièrement dans les cellules adhérentes. C'est lui qui va comprimer le noyau lorsqu'une cellule s'étale. La dépolymérisation des filaments d'actine va regonfler le noyau et favoriser les fluctuations de l'enveloppe nucléaire. Cela a pour conséquence, d'augmenter les mouvements de la chromatine dans le noyau. Il est possible de provoquer la contraction du réseau, ce qui va d'autant plus comprimer le noyau et réduire les mouvements de chromatine à l'intérieur. Ces éléments montrent que la cellule est capable de réguler la forme de son noyau et par la même occasion, d'y influencer l'organisation et la dynamique de la chromatine. Pour aller plus loin, il a même été démontré qu'induire la contraction du réseau d'actine de cellules souches pluripotentes humaines est capable d'induire l'expression de gènes de différenciation.

Il existe d'autres filaments que l'actine capables de déformer le noyau dans une cellule. Ces filaments sont générés à partir d'une structure centrale appelée centrosome. Le réseau formé est une sorte d'étoile entourant le noyau. La contribution des microtubules aux déformations du noyau n'a pas été totalement déterminée mais un nombre croissant d'éléments suggère qu'ils sont bien capables de le déformer mais de manière plus faibles

que l'actine. En effet, en rendant les microtubules plus rigides ou au contraire en rendant le noyau plus souple, des déformations de l'enveloppe nucléaire dues au microtubules peuvent être observées, le plus souvent des invaginations. Dans certaines conditions physiologiques, les microtubules sont capables d'engendrer des fluctuations de l'enveloppe nucléaire et de promouvoir les mouvements de la chromatine. Ces éléments montrent que les microtubules sont également capables, au même titre que l'actine, d'avoir une influence sur l'organisation de la chromatine. Néanmoins, l'impact de ces forces sur l'expression de gènes et l'organisation de la chromatine lors de la différenciation de cellules souches a été très peu documenté.

Afin d'apporter des éléments de réponse à cette question, des cellules non adhérentes, où l'organisation de l'actine est moins propice à déformer le noyau, peuvent être utilisées afin de déterminer la contribution des microtubules dans cette forme. Le système hématopoïétique est constitué de cellules non-adhérentes, et on peut également y trouver une cellule souche: la cellule souche hématopoïétique. Dans le cadre de l'étude de la forme du noyau, il est particulièrement intéressant de noter que les cellules immunitaires innées granulocytaires possèdent un noyau lobulé et il a déjà été suggéré que les microtubules sont impliqués dans ces déformations. Cependant leur impact sur l'organisation de la chromatine n'est pas clair. Pour cette raison, nous nous intéresserons à la différenciation des cellules souches hématopoïétiques dans la lignée myélocytaire (donnant naissance par la suite aux granulocytes), et nous porterons une attention toute particulière à l'architecture intracellulaire, notamment à la forme du noyau et à l'organisation des microtubules.

Afin d'étudier le rôle des microtubules dans l'hématopoïèse, des cellules souches et des cellules peu différenciées (progénitrices) ont été purifiées et séparées en trois populations: les cellules souches naïves, les progéniteurs myéloïdes et les progéniteurs lymphoïdes. La différence principale observée dans la structure intracellulaire est la suivante: les progéniteurs myéloïde possèdent un noyau plus gros et largement déformé (presque lobulé) par rapport aux cellules souches et aux progéniteurs lymphoïdes. Ces déformations sont également corrélées à des distributions d'hétérochromatine constitutive distinctes.

Afin de déterminer l'origine de ces déformations, le cytosquelette des progéniteurs myéloïdes a été analysé. Les microtubules forment des faisceaux épais au niveau des déformations suggérant fortement un rôle des microtubules dans ce phénomène. En cultivant ces

cellules *in vitro* vers la voie myéloïde, il a été possible de reproduire ces observations: après trois jours de culture, le noyau devient plus gros, présente des déformations et une distribution de l'hétérochromatine constitutive comparables à celles observée chez les progéniteurs myéloïdes. La fenêtre temporelle pendant la quelle se produit ce phénomène a été déterminée afin de pouvoir perturber la dynamique des microtubules lors de la déformation et confirmer l'hypothèse selon la quelle les microtubules sont responsable des déformations du noyau lors de la différenciation myéloïde. Par des traitements biochimiques qui perturbent la dynamique des microtubules, en les dépolymérisant ou en les stabilisant par exemple, il a été possible d'empêcher la déformation du noyau. Le plus remarquable étant que, lorsque le noyau n'est pas déformé la distribution de la chromatine n'est pas impactée par rapport à celle des cellules souches.

En résumé, ces travaux montrent que (i) les microtubules sont capables de déformer lors de la différenciation des cellules souches hématopoïétiques dans la voie myéloïde, (ii) la forme du noyau est corrélée à un changement d'organisation de la chromatine, (iii) empêcher la déformation limite la réorganisation de la chromatine observées lors d'une différenciation en condition normale.

De manière générale ces résultats indiquent que les microtubules peuvent être considérés comme des acteurs à part entière de mécano-régulation génétique, au même titre que le cytosquelette d'actine. De plus, il a été montré que des cellules de tumeurs cancéreuses ont une morphologie différente de cellules saines, passant d'un phénotype adhérent à un plus semblablement aux cellules non-adhérentes. Ce changement suggère une réorganisation du cytosquelette, où potentiellement, les microtubules dérèglent l'expression génétique. Les microtubules pourraient devenir ainsi un nouveau angle d'attaque dans les traitements du cancer.

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The terms *stem cell* emerged in the end of 19th century [Ramalho-Santos & Willenbring 2007]. Ernst Haeckel used it to describe the fertilized egg as a cell able to generate all the cell diversity by changing identity of a given organism during embryogenesis [Haeckel 1877]. Embryologists later described the mechanisms governing these identity changes and the terms were used to generally describe cells able to switch identity. The definition then evolved to a more restricted one, the one we know nowadays. Stem cells are indeed able to change identity and give rise to cells of various functions, a process called differentiation, but they are also capable to self-renew, which means they are able to proliferate and keep their differentiation potential, their stemness. This behaviour make them capable to regenerate tissues.

All cells possess the same DNA content, acquisition and maintenance of a new identity thus requires an information encoding gene expression independent of the DNA sequence.

In other words, the question is how can cells change their gene expression profile to acquire new functions ? Epigenetics partially answered this question showing that selective DNA chemical modifications or DNA interactions with proteins could modify the expression/repression state of genes and be transmitted to the descendant. Nevertheless, how the stem cell's identity switch is regulated remains unclear.

It was shown that gene expression could be affected by external biochemical signals via signalling pathways. It is a way for the cell to integrate informations from its environment and adapt. This discovery made it possible to understand how stem cell fate could be oriented towards a specific identity. Interestingly, it was also shown that depending on the physical cues the cell is subjected to, its destiny could be biased: it is the mechanotransduction.

Nowadays it is known that the nucleus, which contains the DNA, is responsive and integrates mechanical stimuli. Modulating mechanical stress on the nucleus can lead to a change of cell identity, mechanical properties of nucleus being the key factors determining the cellular response. The cytoskeleton constitutes the main cellular component able to generate forces and seems to govern mechanically gene expression via its interaction with the nucleus. Altogether it led, step by step, to the hypothesis that modulating the cell's cytoskeleton can impair forces generated on the nucleus, alter gene expression and could thus govern differentiation.

Many questions remain. It is still unclear how gene expression is linked to nuclear mechanical properties. DNA and nuclear envelope must tightly interact so a physical information can go from one to the other. Similarly, how forces applied by the cytoskeleton impact DNA is a topic of growing interest. It could impact DNA integrity and/or organization but how it would affect gene expression remains poorly described. In this work I will describe the current knowledge on how stem cells fate can be controlled, how physical constraints can contribute to fate determination, especially how cytoskeleton organization and its dynamics are involved in this phenomenon.

I Stem cells

1 Definition

Stem cells are found in all multicellular organisms and are needed for their homeostasis, i.e. the maintenance of the integrity of its tissues over a long period of time. In other words, it means that they are able to maintain and adapt organs' function to changes in physiological or pathological conditions [Majeti *et al.* 2007] [Notta *et al.* 2015]. Skin and blood are the most intuitive examples. In physiological conditions epidermal cells or red blood cells have a limited life span because of the constant friction they're subjected to (external for skin, squeezing in vessels for red blood cells). These events, among others, damage the cell and will impair their functions. The damaged cells then have to be replaced, that's where the stem cell comes into play and will generate new cells. In some cases, it happens that an organism must adapt to a new condition, like gestation. Here, the added necessity of vascularizing an embryo leads to a proliferation of hematopoietic stem cells in the mother in order to increase the amount of blood available [Nakada *et al.* 2014]. Similarly, in pathological conditions, such as bacterial infection, the number of immune cells has to be increased in order to prevent bacterial growth. Here again the hematopoietic system is stimulated to increase the number of immune cells available. Nevertheless, the system is not always able to sustain proper functions, as it can be observed during aging, where stem cells ability to restore the correct amount of blood cells is reduced [Shlush *et al.* 2015].

1 Hematopoietic stem cells during development

In mammals, two types of stem cells can be found: the embryonic and the adult stem cell. Embryonic stem cells (ESC) are pluripotent cells, which means they can generate what are called the three germ layers: the endoderm, the mesoderm and the ectoderm. Together, the germ layers generate all the tissues of the embryo although they cannot sustain the complete development of an embryo by themselves, they do not produce annexes like the placenta. These cells can only be found in early stages of development in a region called the inner mass (figure 1.1).

During development, stem cells of a given tissue will be localized at several specific sites. For instance human hematopoietic stem cells (HSCs), historically the first ones discovered,

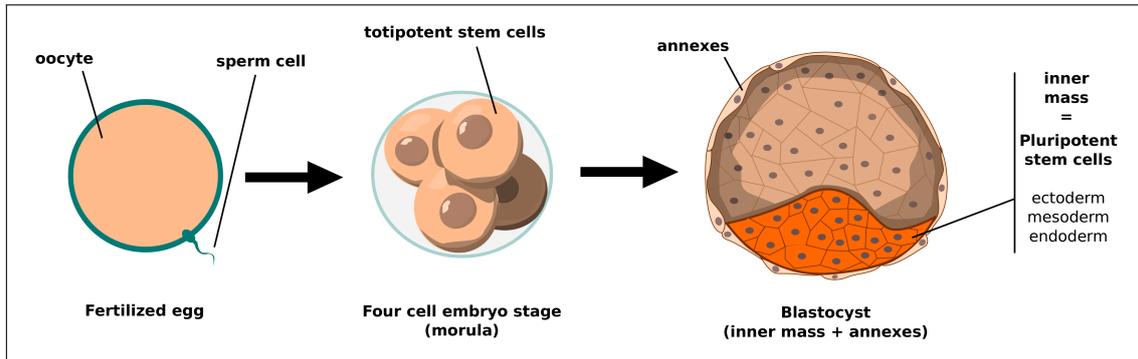


Figure 1.1 – After fertilization, the egg undergoes several mitotic events and generate the blastocyst. There, ESC can be found in the inner mass, outer cells will form the annexes like the placenta.

are located in different organs during development. Schematically they start in the yolk sac, then simultaneously in the liver and the spleen to finally locate in the bone marrow, in the adult [Mirshekar-Syahkal *et al.* 2014]. During these transitions, the activity of HSCs varies. The liver and the spleen are hosts of a huge expansion period, during which HSCs mostly self-renew. Then, when moved to the bone marrow, mature enough, starts the regulation to maintain blood functions in the adult [Mirshekar-Syahkal *et al.* 2014]. This observation highlights the importance of extracellular signals in hematopoiesis regulation. Cells are the same, but according to their location behave differently, suggesting that their environment, contributes a lot to their regulation.

2 Adult hematopoietic stem cells

The bone marrow, the adult HSCs location, is located in the bone epiphysis and is highly vascular. As a consequence, it possesses two distinct environments, one associated to the bone and the other to the blood vessels. These two regions communicate via soluble factors and cells, mainly mesenchymal stem cells (MSCs) [Méndez-Ferrer *et al.* 2010]. The bone side, composed of osteoblasts, forms the endosteal niche, and the vascular side, composed of endothelial cells, forms the perivascular niche. Both sites assume different functions, the first one locates the quiescent stem cells and the other one the active ones [Ehninger & Trumpp 2011]. In the end, the niche forms a highly complex environment made of various cell types able to regulate the activity of HSCs. The role of the micro-

environment becomes a growing topic of interest in the stem cell field and will be described later.

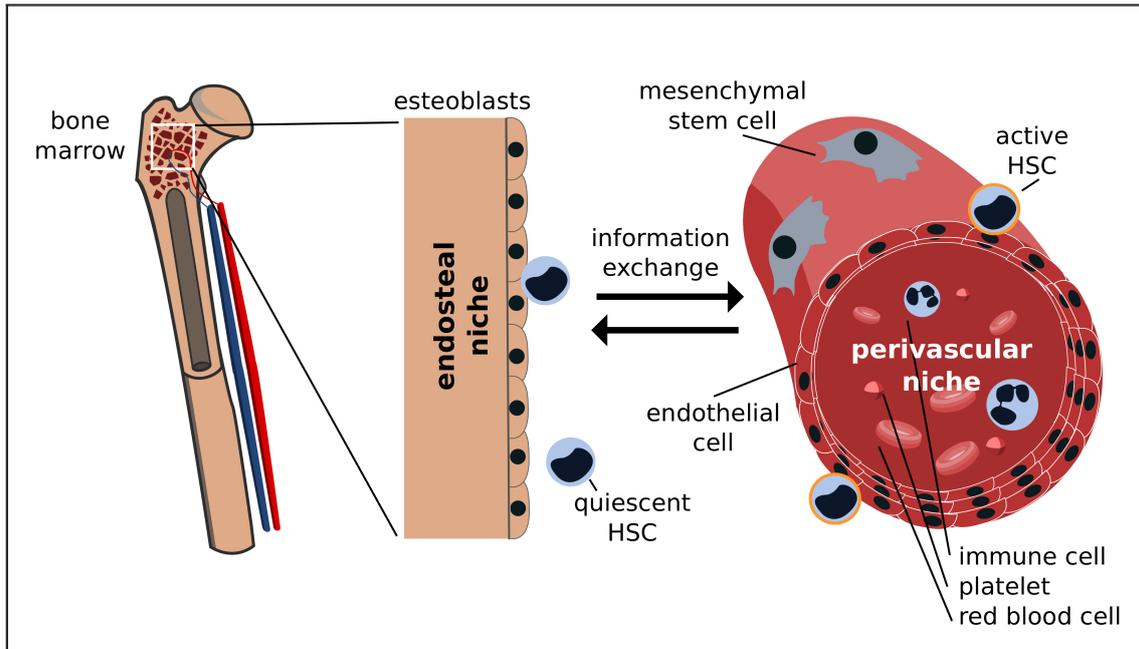


Figure 1.2 – Hematopoietic stem cells are located in bone marrow. The stem cell can interact with two distinct environments: the endosteal one formed by osteoblasts and the perivascular niche formed by endothelial cells. According to their location they will be quiescent or active.

As said previously, the potency of stem cells finally stabilizes at the adult stage. At that stage, HSCs differentiation potential can be quantified experimentally in terms of colony forming units. What it means is that depending on the stage of differentiation when extracted from the organism, the cell (named colony forming cell (CFC)) is able to generate colonies on agar plated Petri dishes of specific cell types [Sarma *et al.* 2010]. Analysis of surface proteins, which are membranous proteins grouped under the name of cluster of differentiation (CD), of a given colony made it possible to link this differentiation potential to a molecular identity [Terstappen *et al.* 1991]. From there, it became possible to sort hematopoietic cells in order to purify stem cells and study their behaviour [Hogan *et al.* 2002]. It led to the classical hierarchical step-like vision of the hematopoietic system (figure 1.3).

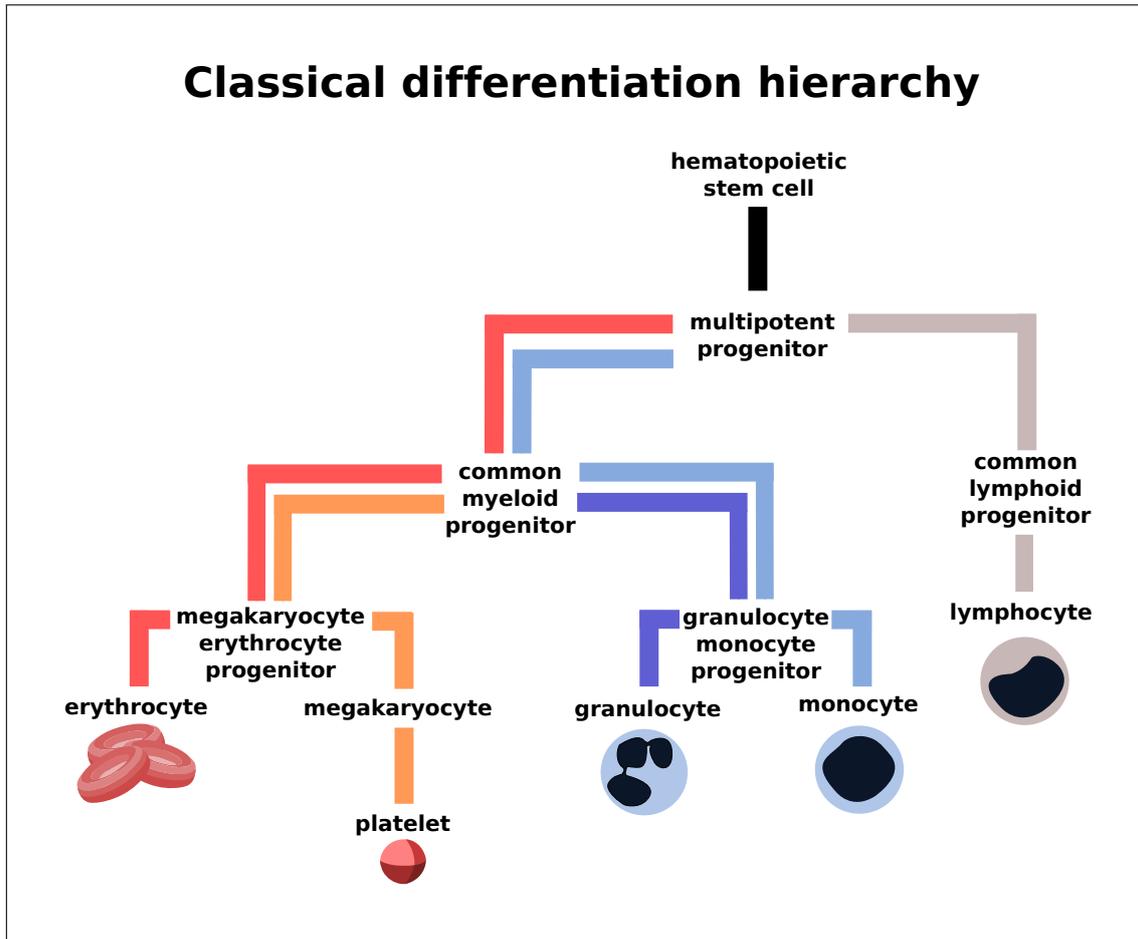


Figure 1.3 – Simplified classical view put the stem cell as the root of a tree, which nodes represent defined intermediate states (progenitors) that can generate specific cell types. The end of the branches are the terminally differentiated cells (Adapted from [Notta *et al.* 2015]).

3 Organ homeostasis and therapeutic potential

Two behaviours characterize the stem cell activity and are crucial for organs' homeostasis: self-renewal and differentiation. Self-renewal means the cell, upon symmetric division, generates two equivalent daughter stem cells, leading to an increase of the population pool of stem cells. On the contrary differentiation is the process during which the cell acquires a new identity, usually happening through asymmetric division.

Balance between self-renewal and differentiation is tightly regulated. On the one hand if cells divide too much, this could lead to a tumor-like behaviour, on the other hand if the cells differentiate without self-renewing it will diminish the pool of stem cells, to the point of total depletion and organ dysfunction because it can't regenerate at all. It is what happens

when stem cells are extracted and kept out of their physiological environment (*ex vivo*): they tend to differentiate spontaneously without self-renewing, even though already differentiated cells proliferate and increase to total number of cells [Capellera-Garcia *et al.* 2016] [Roch *et al.* 2015].

Stem cells, due to their regenerative potential are promising for cellular therapy. Grafting stem cells is a possible way to regenerate damaged or non functional organs. Historically the first cell therapy was developed with the hematopoietic system in 1956 with the first bone marrow transplants [Thomas *et al.* 1957]. After bone marrow destruction with radiations or chemotherapy, transplantation of a healthy bone marrow containing HSCs can regenerate a functional hematopoietic system. It is nowadays still the main treatment for blood related cancers. Unfortunately, immunosuppressing treatment is then needed, as for any grafts. The treatment presents other limitations. First healthy HSCs harvesting requires bone marrow donation which is not an easy procedure and the number of donors is small, secondly, due to the low number of collected cell, only a low number of stem cells will be present and it will reduce to the graft success rate. One way to resolve the problem is to expend the few available stem cells by engineering *ex vivo* culture conditions in which the stem cells proliferate without differentiating [Zonari *et al.* 2017]. Another way is to have a more accessible source of stem cells, like umbilical cord blood, which contains circulating foetal HSCs. This technique is non invasive and more reliable due to a more or less constant number of deliveries. Nevertheless, donors compatibility is still required. To overcome this issue, most recent research focuses on the use of embryonic stem cells or induced pluripotent stem cells to generate HSCs. It would increase the amount of cells available and suppress any immunological constraint, in the latter case [Daniel *et al.* 2015] [Choi *et al.* 2011] [Lachmann *et al.* 2015].

2 Stem cells regulation

1 Acquisition of a new identity

The most extensively studied mechanism of stem cell differentiation is the asymmetric stem cell division. As its name suggests, this process gives two different daughter cells after mitosis. One possibility to obtain two different cells is that the cell is polarized before

division. The cell can indeed segregate proteins to a given location that will induce an asymmetric phenotype. Then, during mitosis, spindle orientation will determine the content of the daughter cells: if perpendicular to the polarization axis, the cell content will be different between the two cells, if parallel to the polarization axis, the two daughter cells will be equivalent. The main model of asymmetric stem cell division is the drosophila neuroblast. This stem cell is able to polarize what is called a cell fate determinant, the Numb protein in this case. This protein is known to be involved in the maintenance of stemness by repressing a specific subtype of genes, if not present the cell will differentiate. Via Numb polarization and asymmetric division the neuroblast cell will generate two different daughter cells, one neuroblast and one ganglion mother cell (GMC) that will then form neurons [Roubinet & Cabernard 2014] [Gómez-López *et al.* 2014].

These differences of content will induce differential gene expression thus an identity switch. In stem cells repressing certain type of genes, and expressing others will participate to the maintenance of the stemness [Atlasi & Stunnenberg 2017] [Liu *et al.* 2016] [Peric-Hupkes *et al.* 2010] [Petruk *et al.* 2017] [Ugarte *et al.* 2015]. In other words, specific genes are expressed in the stem state and if not, cells tend to differentiate [Lopes Novo & Rugg-Gunn 2016].

Maintenance of gene expression profile through several generations happens via an ensemble of biochemical modifications either of the DNA itself or proteins associated to it. These modifications are referred to as *epigenetics* (greek prefix *epi-* which means ‘on top of’, here on top of genetics). The most studied protein post-transcriptional modifications are those of histones which form together with the DNA, the chromatin. All these sequence-independent modifications will participate to the compaction (heterochromatin) and decompaction (euchromatin) of the chromatin [Lopes Novo & Rugg-Gunn 2016]. When chromatin is not compacted, it is accessible to the transcription machinery responsible for gene expression and on the contrary, compacting it silences it. In the end modulating the number of histones and the chemical functions responsible for their interaction with the DNA allows to tune gene expression [Atlasi & Stunnenberg 2017] [Farlik *et al.* 2016]. It appears that histones modifications in stem cells are unstable when stem cells are extracted from their physiological environment which could explain the impossibility to keep the stem state *ex vivo* [Rajamani *et al.* 2014]. Today’s biggest challenge of the stem cell field is to

understand how the balance between self-renewal and differentiation is controlled at the chromatin level, and how these conditions can be reproduced in a Petri dish.

Going in this direction, 2012's Nobel Prize, Shinya Yamanaka, has shown that mature cells could be reprogrammed into cells with stem cell capabilities only by inducing a small amount of genes [Takahashi & Yamanaka 2006] and were named induced pluripotent stem cells (iPSC). This founding highly contributed to the concept of stem genes. Then has emerged the idea that differentiation, previously described by discrete well defined steps is now seen as a continuous process, where these steps are virtual, each gene expression profile variation potentially being a new step [Macaulay *et al.* 2016] [Velten *et al.* 2017]. Supporting this view, analysis of single cell gene expression profiles showed that what was thought to be a unique specific stage, stem cells can be represented by a heterogeneous population. This result shows that a single stem state is not definable. Most probably it is even irrelevant. The tree-like classification illustrated with HSCs (figure 1.3) where each node is a common precursor for other cell types now seem obsolete. Although this representation remains correct, it is not accurate. Illustrating this knowledge shift, experiments have shown that, differentiated cells could arise directly from stem cells without going through the known intermediate states and without divisions [Roch *et al.* 2015]. The current hypothesis is that stem cells are a reservoir common to several lineages and that during early steps, differentiation can be reversed to another lineage [Notta *et al.* 2015] (figure 1.4).

These results strongly suggest that the stem cell state is not unique and that differentiation modalities are numerous. Under specific conditions cells can change their fate, a stem cell can jump “steps”, differentiate without dividing, and a mature cell can even reacquire a stem cell behaviour. Nowadays, the main goal is to understand how these different balances are regulated; i) the self-renewal/differentiation one and ii) the one between the differentiation paths, determining which mature cell type will emerge from a single stem cell.

2 Controlling cell identity: the biochemical way...

Gene expression is controlled via transcription factors, that are proteins interacting with promoter sequences of a gene to activate its expression. Transcription factors activation

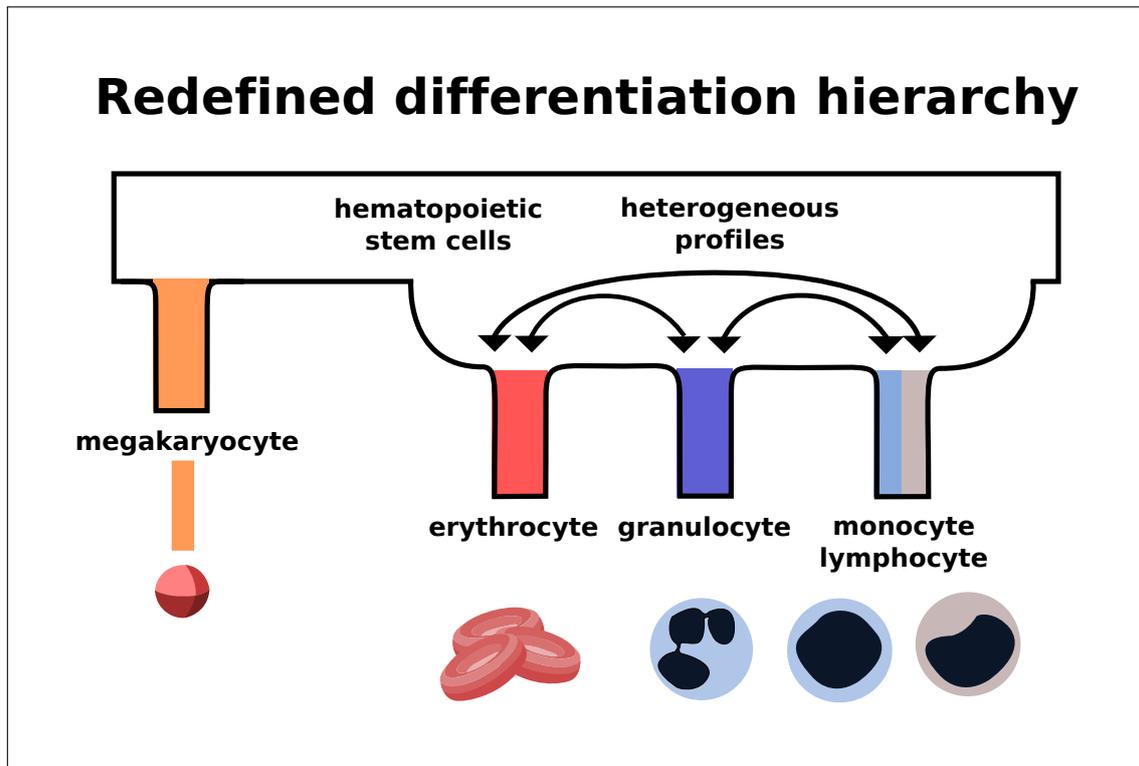


Figure 1.4 – Stem cells are now seen as a heterogeneous population that can enter differentiation paths. These paths have to some extent common progenitors and are entangled. Lineage specific differentiation can even directly occur from the stem cell without intermediates. (Adapted from [Notta *et al.* 2015])

is tightly regulated by signalling pathways which allow to transduce a message from the extracellular medium up to the DNA. This process works as follow: a biomolecule, either soluble or present on another cell's surface, interacts with a cell's membrane receptor through its outer domain. This receptor will change conformation exposing new interaction sites for cytoplasmic proteins. So is triggered an activation cascade: activated proteins by the receptor will in turn activate new ones, and so will the newly activated proteins, amplifying the initial signal. The final effector is a protein, the transcription factor regulator. This protein will be translocated into the nucleus via nuclear pore complexes (NPCs) so it can interact with its transcription factor in order to affect chromatin transcription state (figure 1.5).

Historically most activation pathways were discovered in the fruit fly *Drosophila melanogaster* by Christiane Nüsslein-Volhard and Eric Wieschaus rewarded by a Nobel Prize in 1995. Many pathways are involved and are crucial for correct organisation of the

embryo (segmentation, bilateral symmetry...) like Wnt/Beta-catenin, TGF-beta/ SMAD, BMP/SMAD and Notch [Watabe & Miyazono 2009]. Later, during the adult stage, same pathways are involved in the organism homeostasis and particularly in stem cells regulation. For instance it has been shown that Wnt as well as Notch are involved in hematopoietic stem cells self-renewal regulation through the activation of the transcription factors OCT4 and Nanog [Blank *et al.* 2008] [Katoh & Katoh 2007]. These pathways form a highly complex network, with common effectors or common receptors, making the combination of all of them difficult to decipher.

One of the biggest achievements of the past decades has been to develop different cell culture medium inducing different types of differentiation *ex vivo* [Berger *et al.* 2002] [Donaldson *et al.* 2001]. They use standard medium supplemented with soluble factors called cytokines to activate or repress specific signalling pathways regulating stem cell fate.

3 ...and the mechanical way

Cells are able to respond, not only to chemical signals, but also to the physical ones, like environment physical properties or physical constraints. It is called mechanotransduction. To be more precise, mechanotransduction is the ability of a given cell to integrate a mechanical information from its surrounding and transduce it to the chromatin, converting a mechanical information into a biochemical signal capable of affecting chromatin.

The biggest discovery in the field of mechanosensing was made when mesenchymal stem cells (MSCs) were left to differentiate on substrates of different stiffnesses. Cells adopt different shapes depending on the stiffness of the substrate thus influencing intracellular organization. If the biochemical environment is kept constant, MSCs fate could be oriented towards osteogenesis by putting them on a stiff substrate, or towards adipogenesis by putting them on a soft substrate [McBeath *et al.* 2004]. This result shows that mechanotransduction is a processes capable of driving gene expression and impact differentiation.

Two transcription factors, YAP and TAZ have been shown to localize in the nucleus according to the cell physical environment [Piccolo *et al.* 2014]. These two factors are part of the Hippo signalling pathway that regulates organ growth by controlling cell prolifer-

ation. The mechanism by which they get shuttled to the nucleus remains unclear but it is known that physical cues and not biomolecules trigger their relocalization. When cell environment does not allow spreading, like during confluence, YAP and TAZ are relocated from the cytoplasm to the nucleus. These two transcription factors act thus as sensors of the physical environment [Low *et al.* 2014]. It is a mechanism involved in skin differentiation, as during epithelial growth cells change their neighbouring contacts. According to a cell position in the epithelium, in contact with the basal membrane (separating the epithelium from the underneath connective tissue) or not, YAP/TAZ will control skin stem cells activity and participate to its homeostasis [Elbediwy *et al.* 2016]. All these shape changes correlate strongly with the actin cytoskeleton organization which defines the framework of the cell. It led to the discovery that disruption of actin filaments can impair YAP/TAZ localization [Low *et al.* 2014] showing that the cytoskeleton is necessary for the cell to sense correctly its physical environment and thus connects the mechanical cues the cell receives to biomolecular effectors.

Actin is capable of gene expression modulation via another modality. It has been shown that soluble actin can directly interact in the cytoplasm with a transcription factor activator, MAL. When actin reorganization is triggered (via mechanical cues like the ones described previously), soluble actin will unbind MAL. MAL, now free of actin, can shuttle into the nucleus, interact with its target, the serum response factor (SRF), a transcription factor. SRF is involved in many developmental processes, such as muscle growth or cardiac muscle differentiation [Miralles *et al.* 2003].

Presence of soluble actin itself inside the nucleus has also been demonstrated [de Lanerolle 2012]. This pool of actin seems to be able to modulate transcription by interfering with RNA polymerase activity [de Lanerolle 2012]. The mechanism of this regulation is still to be understood, the complex formed by actin and other associated proteins (like myosin) with the transcription machinery couldn't be clearly described yet.

Actin is capable of regulating transcription factors activity, directly or indirectly, and is also involved in cell shape which depends on external cues, it becomes a major component for mechanotransduction and makes it an important actor in stem cell fate regulation [Driscoll *et al.* 2015].

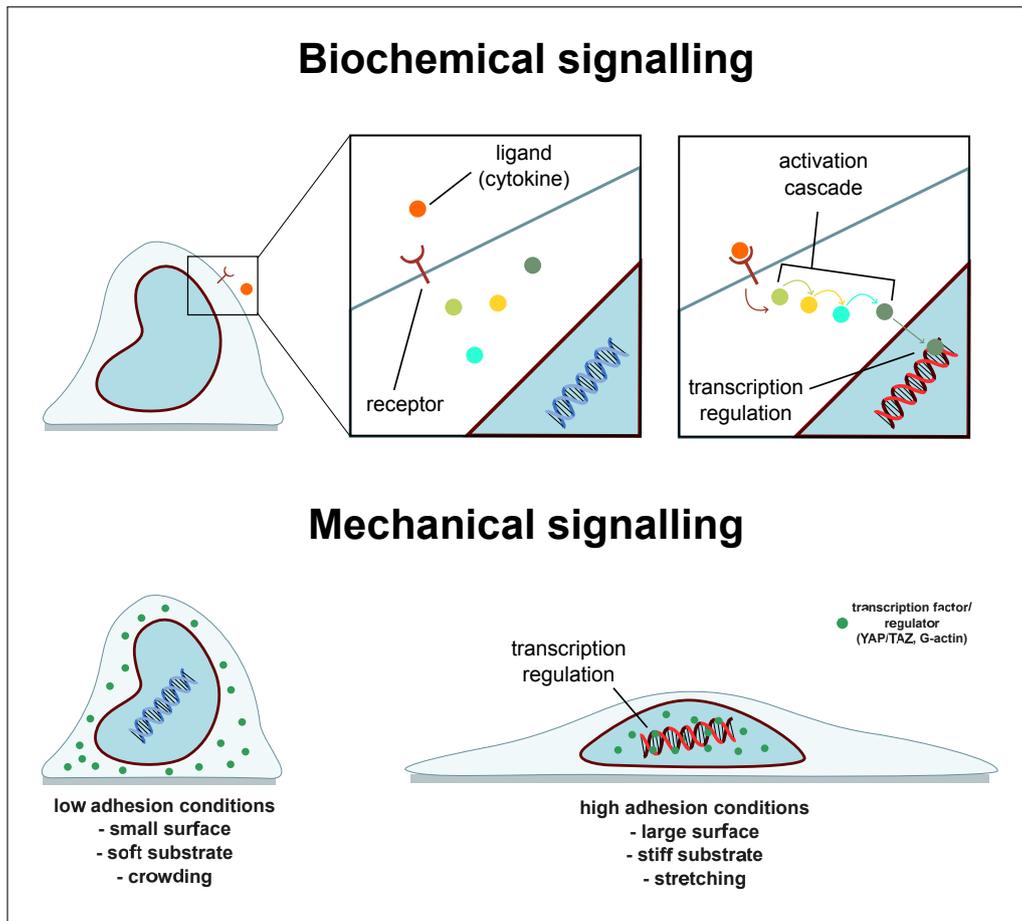


Figure 1.5 – Biochemical signalling goes through soluble molecules binding a receptor. This receptor will induce an activation cascade ending to the translocation of a transcription factor in the nucleus that will regulate gene expression. Mechanical signalling allows the cell to sense physical cues. When the cell adheres, cell shape changes, induces intra cellular architecture reorganization enabling translocation to the nucleus of transcription factor regulators.

3 Current Issues

Many questions remain concerning the stem cell behaviour. The main one being the control of self-renewal vs. differentiation. Understanding this balance would make possible to maintain and expand stem cells in culture and make more efficient cell therapies. It should be noted that this limitation has been partially solved with ESCs. Indeed, with all the signalling pathways discovered and the development of synthetic cytokines it is now possible to keep their stemness *ex vivo* and protocols exist to differentiate them into the desired cell types [Lim *et al.* 2013] [Karakikes *et al.* 2014] [Ben-Shushan *et al.* 2015] .

To further understand how this balance is regulated it is necessary to go through the study of the interplay between biochemical and mechanical pathways controlling differentiation. It means that the whole environment has to be taken into account. Nowadays, many researches focus on this topic and led to the concept of the stem cell niche (also known as micro environment). The stem cell cannot be considered only as an isolated cell, but exists within a certain context. The most striking example of a niche is the drosophila testis. In this system, germline stem cells are organized around hubs which are small clusters of somatic stem cells. The germline stem cells divide asymmetrically, one daughter cell moving away from the hub and differentiate, the other one keeping contact with the hub. The cell in contact with the hub generates membrane protrusions called nanotubes. These nanotubes create invaginations in a hub cell resulting in an increased surface contact, enriched in signalling pathways receptors. If the daughter cell loses this specific contact it loses its stemness and differentiate but can recover it if it is back in contact with the hub [Inaba *et al.* 2015]. Similar results were obtained in the airway system, where progenitors daughter cells are able to prevent their progeny differentiating by keeping a physical contact with them [Pardo-Saganta *et al.* 2015].

To investigate even more the role of the niche, organoids, sometimes called mini organs, were developed. They are small aggregates of different cell types reproducing multi cellular cell-to-cell interactions. These multicellular structures are generated from embryonic stem cells and reproduce to a certain extent an organ architecture with all the physiological cell diversity, including the stem cells, hence the name. The stem cell can be studied in its environment, and many results suggest, as did the iPSC discovery and the drosophila testis model that indeed, in the absence of stem cells in the niche other cell types could do the job, i.e. can reacquire a stem cell identity [Tata *et al.* 2013]. Different fields are needed to tackle stem cells regulation question properly, from biochemistry to integrated tissue biology going through cellular biology.

Another critical point to understand stem cells stability concerns the DNA itself. How can the cell identity be conserved over generations ? How DNA is modified to impact cell fate ? The explanation goes through the study of the organization of the chromatin inside the nucleus and current knowledge on this topic is detailed in the next section.

II Chromatin and nucleus

1 Chromatin organization

1 Chromatin structure

Chromatin structure was already briefly addressed in the previous part. Two different types of chromatin can be distinguished according to their condensation state. Either decompacted (euchromatin) or compacted (heterochromatin). Histones are small proteins, highly conserved through evolution, forming octamers (2xH2A, 2xH2B, 2xH3, 2xH4), around which the DNA will be wrapped, and maintained by a histone H1, participate to chromatin condensation. The complex formed is called the nucleosome. Forming more or less nucleosomes, DNA becomes more or less compacted, thus less or more accessible to proteins like transcription factors, RNA polymerases etc. In order to modulate chromatin compaction state, specific amino-acids can be enzymatically modified

on a specific histone like the tri-methylation (me3) of lysine 9 (K9) of the histone number 3 (H3): H3K9me3. The most thoroughly described histones modifications are (de)methylation (made by Histone Methyl Transferase) and (de)acetylation (made by Histone Acetylase (HAT) and Histone DeAcetylase (HDAC)) and seem to be the main ones involved in differentiation although many others exists (sumoylation, ubiquitination...). These modifications have different roles, some methylation, or acetylation, will be associated to repressed genes, others will be associated to active ones

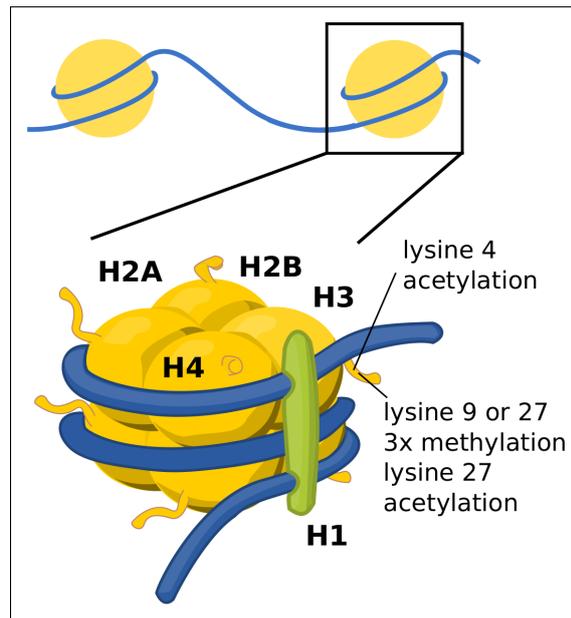


Figure 1.6 – DNA is wrapped around histones forming the nucleosome. The N-terminal tail of each histones can be modified. Most important ones for gene repression and expression in stem cells are represented. Adding more or less histones will lead to condensation of the chromatin making it less accessible to transcription factors.

[Atlasi & Stunnenberg 2017]. Moreover, these epigenetic marks are known to evolve during differentiation. Genes known to have modifications in the stem state, such as Nanog target genes, lose them over lineage commitment. These epigenetic marks are generally associated to decondensation, when lost they can trigger repression of stem state genes, leading to differentiation [Lopes Novo & Rugg-Gunn 2016]. Put in other words, decondensation state of the chromatin seems to be characteristic of the stem cell state [Talwar *et al.* 2013]. Almost twenty years ago, the concept of a histone code was proposed [Strahl & Allis 2000] but some functions associated to marks seem to be species, tissue or even gene specific and make the identification of such a code inapplicable.

2 Chromatin organization in the nucleus

In the nucleus, chromatin will interact with proteins constituent of nuclear envelope leading to a highly 3D organized chromatin filament. The highest level of compartmentalization, and historically the oldest, is the chromosome territory proposed in 1885 by Carl Rabl (figure 1.7). Before that, nucleus and chromatin were thought to be like a spaghetti bowl, where DNA was intermingled randomly in the nucleus. Chromosome painting techniques (like Fluorescence in situ Hybridization (FISH) using complementary DNA sequence fluorescent RNA sequence) have shown that the localization of gene loci depends on their activation state: active, they locate far from repressed regions, whereas inactivated they come closer [Francastel *et al.* 2000]. It gave birth to the idea that chromatin regions of similar condensation state cluster together and that these compartments are dynamic. Then, techniques of chromosome conformation structure capture were developed and gave a way more precise and global picture of DNA organization. The technique consists of fixing DNA with paraformaldehyde so sequences spatially close to each other will be cross-linked. Then DNA is digested and ligated so linked sequences will form only one. Sequencing of the resulting fragments then allows to create a map of DNA-DNA interacting sequences. Results strengthened the chromosome territories idea, showing that most of the inter chromatin contacts happen inside a single chromosome [Wang *et al.* 2016]. Data analysis became more and more powerful over time and led to a fine description of the interactions leading to the discovery of topologically associating domains (TADs) [Dekker & Mirny 2016]. These are active regions physically isolated from others via a loop extrusion mechanism.

These loops are formed by an annular protein called cohesin, that bind chromatin and pull on each of its side generating a loop that will go through the ring shaped protein (figure 1.8). Loops are limited by transcriptional repressing factor CTCF (also known as CCCTC-binding factor) creating boundaries of the active regions. These loops are thought to be the basic building blocks for 3D chromatin organization, and also that cell type specific functions could be achieved by their combination. Nevertheless, CTCF depletion alone does not seem sufficient to disrupt TADs' structure in every cases [Hou *et al.* 2010] [Barutcu *et al.* 2018].

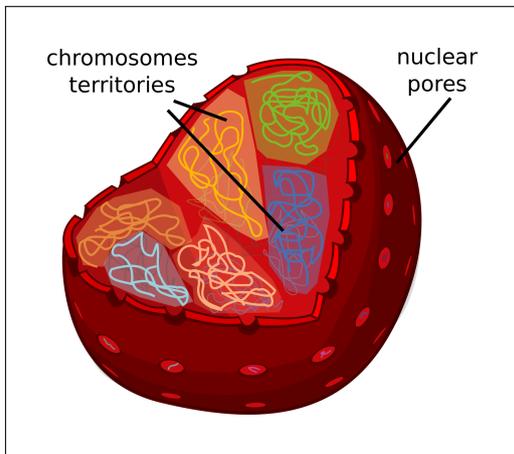


Figure 1.7 – Most of DNA-DNA interactions happen inside the same chromosome meaning each chromosome interacts less with its neighbours.

Active (non-condensed), and inactive (condensed) regions are distributed relatively to each other following a certain pattern. Electron microscopy techniques made possible to see eu- and heterochromatin positioning in the nucleus and showed the now acknowledged, peripheral heterochromatin vs. central euchromatin distribution [Francastel *et al.* 2000]. This observation supposes that specific sequences or elements associated with repression of the genome can interact with the nuclear envelope.

3 Chromatin interaction with nuclear envelope

The nuclear envelope is formed of two lipid bilayers, covered on the inside by a network of filaments forming the lamina. In mammals the lamina is composed of the lamin proteins, which are part of what are called intermediate filaments (IFs), members of the cytoskeleton family. This class of filaments can be found also in the cytoplasm and are thought to be relatively static and to give it its visco-elastic properties [Chernyatina *et al.* 2015]. Every types of intermediate filaments have the same structure: two filaments will associate in a parallel fashion, then, this dimer, will form a tetramer in which two dimers are assembled in an antiparallel way. The tetramer is the fundamental unit of the IF and will polymerize to form a meshwork.

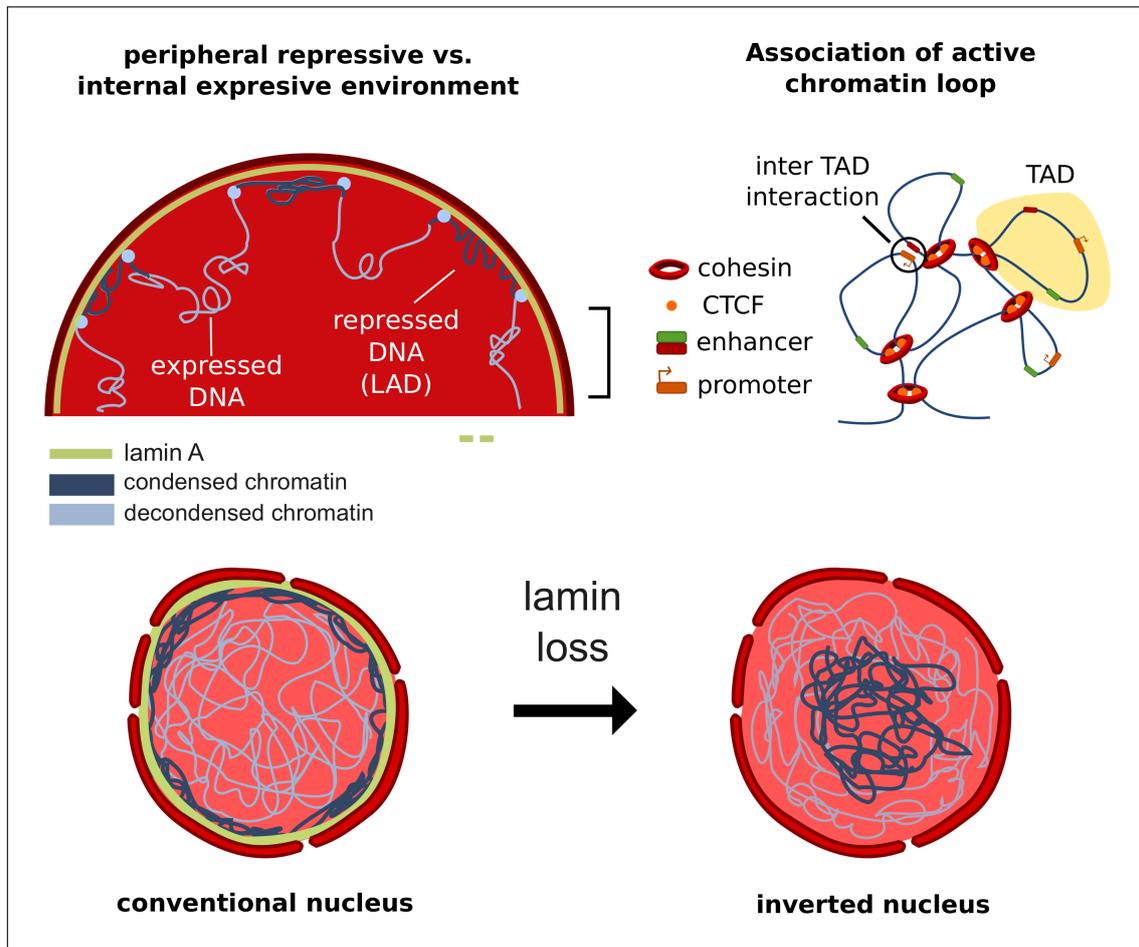


Figure 1.8 – Chromatin usually localizing at the periphery associates with lamins forming lamin associated domains (LADs) and correlates with repressive chromatin marks (heterochromatin). On the contrary chromatin located in the center is often associated with expression marks (euchromatin). Expressed regions are organized in transcriptional active domains (TADs) associated together. If interaction between chromatin and lamin is removed, heterochromatin spontaneously positions in the central part.

Chromatin regions interacting with the lamina were named lamina associated domains (LADs) and can be identified with the DamID technique (DNA Adenine Methyltransferase Identification) [Guelen *et al.* 2008] [Kind *et al.* 2013]. This technique uses the adenine methyltransferase (AMT) present in bacteria but not in eukaryotic cells. The protein of interest, is fused to this enzyme, so if an interaction between the protein of interest present in the lamina and chromatin happens, AMT will methylate the adenines of the DNA, thus making it possible to identify the binding sequences and map them. Using this technique, it has been shown that the regions interacting with lamins are usually poor in

active genes and bordered with CTCF interacting domains (also present on TADs boundaries) meaning these regions are clearly defined and selectively clustered at the envelope.

On top of the DNA sequence, histone modifications could also be characterized. LADs are strongly associated to repressive histone modifications [van Steensel & Belmont 2017]. This again, participate to a highly compartmentalized chromatin vision, where active and inactive regions position is tightly regulated. As it was made for epigenetic marks [Atlasi & Stunnenberg 2017], LADs evolution was analyzed during lineage commitment [Peric-Hupkes *et al.* 2010] [Cesarini *et al.* 2015]. The results show that some genes move away from the lamina to be activated, but not all. Some remain inactivated and it is thought that they are prepared for further activation during the next steps of differentiation. Similarly, preventing repressive marks appearance prevent peripheral relocalization [Towbin *et al.* 2012].

In other models, like trypanosoma, similar repositioning can be observed. Loci of the variant surface glycoprotein (VSG) and procyclin, are expressed in different contexts, respectively during mammal infection and during insect infection. VSG are highly variable glycoproteins positioned at the surface of the cell, needed for the trypanosoma to escape host immune system by increasing presented antigens variability. Many copies of VSG are present on different chromosomes and are located in telomeric regions. Interestingly a single organism expresses only one VSG during its lifetime meaning all the other copies are inactive. Repression of these regions occurs by including these them to the peripheral heterochromatin. On the contrary the active loci are moved to a region called the expression body site (EBS) [Field *et al.* 2012] far from the periphery. Similar regulation is observed for procyclin [Field *et al.* 2012]. These results show that the correlation between chromatin positioning and gene expression is a highly conserved mechanism in eukaryotic cells.

To decipher the contribution of decondensation and activation on positioning, it is possible to express artificially in a cell a synthetic transcription factor which has a specific peptide sequence that induces chromatin decondensation but lacks the transcriptional activation function. If targeting this synthetic transcription factors to a gene sequence it is then possible to compare the effect of mere decondensation to complete activation, on chromatin positioning. With this technique it was shown that decondensation induces comparable gene relocalization to the centre of the nucleus than the one observed during

physiological differentiation. It shows that decondensation and transcription effects on positioning can be decoupled. Condensation of the chromatin alone can affect its interaction with the nuclear envelope and govern its positioning [Therizols *et al.* 2014]. The lamina then appears as one of the important actor in chromatin organization and gene expression.

4 Nuclear periphery vs. nucleoplasm ?

Several studies tend to moderate this well established active-centre vs. repressive-periphery view. Nuclear Pore Complexes (NPCs) make a connection between the inside and the outside of the nucleus. Through them, exit the newly transcribed messenger RNAs (mRNAs) that will be translated into proteins. It has been demonstrated that chromatin can interact with the NPCs and can induce gene transcription [Pascual-Garcia & Capelson 2014] [Ptak *et al.* 2014]. Similarly, in plants gene activated by light exposure are relocated at the nuclear periphery [Feng *et al.* 2014].

The centre of the nucleus does not seem to be only reserved for active genes. Indeed, all lamins are not polymerised in the lamina. Some are present in a soluble form in the nucleoplasm. The same way it interacts with DNA at the inner nuclear membrane, some evidence suggests that soluble lamins can also interact with DNA and affect its expression levels [Lund *et al.* 2013].

Giving importance to this soluble part, some experiments gave counter intuitive results. Lamins have been shown to participate to polycomb group proteins (PcG) complexes integrity. PcG are protein complexes involved in gene repression by forming visible foci in the nucleus which are known to be involved in myogenic differentiation for instance [Cesarini *et al.* 2015]. When lamins expression is impaired, these foci are more dispersed and disassemble [Cesarini *et al.* 2015]. Going in the same direction, upon lamins depletion, telomeres, non expressed chromatin regions, have increased mobility inside the nucleoplasm [De Vos *et al.* 2010]. These two examples show that lamins depletion can have long range effect, in the nucleoplasm, although at first they locate mostly at the periphery. Further study of lamin depletion will have to take into account the contribution of the soluble part.

Questioning even more the necessity of the heterochromatin peripheral positioning, there exists a cell type, the rod cell of the rodents' retina, that exhibits an 'inverted' nuclei, meaning the heterochromatin is located in the central part of the nucleoplasm

[Eberhart *et al.* 2013] (figure 1.8). Mathematical models tried to describe this observation based on chromatin physical properties (mobility, capabilities of interaction). Modelling, although theoretical, has the advantage to simplify drastically a system and allows to tune freely parameters and see if they can explain current observations and can also lead to new hypothesis. Models dealing with chromatin consider the chromatin as a pearl necklace, i.e. beads linked together, and subjected to Brownian motion. A model in which only the mobility of chromatin, dependant of its condensation state and of nuclear envelope oscillation, is able to produce spontaneous heterochromatic regions positioning in the central part of the nucleus [Awazu 2015]. A similar model, based not on motility but on interaction between different types of chromatin and between the chromatin and the lamina lead to the same results [Falk *et al.* 2018]. In this case, the difference of interaction between different chromatin states is not fully established. These results raise the question of the consequences of local lamin disruption. If global lamin loss is able to ‘invert’ a nucleus, local loss should move heterochromatin at the center as well. Thus, if lamin organization can be locally modified via external forces for instance, it could impact chromatin positioning and expression.

5 Towards a mechanical regulation of chromatin organization

Altogether, this data underline the fact that the chromatin is highly compartmentalized in the nucleus. Even though first observations tend to establish a simple pattern (periphery vs. centre), exceptions make it difficult to establish a rule of thumb between position and expression state. More studies of the chromatin organization are needed to be able to draw a global picture.

One could think that forces on the nuclear envelope could impact the compartmentalization by modulating chromatin compaction state or by increasing its movements for instance. Similarly, because heterochromatin is tightly associated to lamina, disruption of lamins could affect its interaction with chromatin and nuclear integrity. These two aspects lead us to explore the mechanical properties of the nucleus and to investigate how they are linked to chromatin organization and gene expression.

2 Nuclear mechanics

To probe more or less directly mechanical properties of the nucleus, different techniques can be used, mainly micro-pipette aspiration or atomic force microscopy (AFM) [Haase *et al.* 2016] [Guilluy & Burrridge 2015]. Unfortunately, these techniques lead to variable results depending on how measurements are made and on which cell type [Dickinson *et al.* 2015] and does not allow to determine an absolute nuclear rigidity value. Nevertheless, they are still able to show that the nucleus is much stiffer than the cytoplasm, and is probably the stiffest organelle of the cell. Nuclear sturdiness has several origins, the main ones being the chromatin and the lamina.

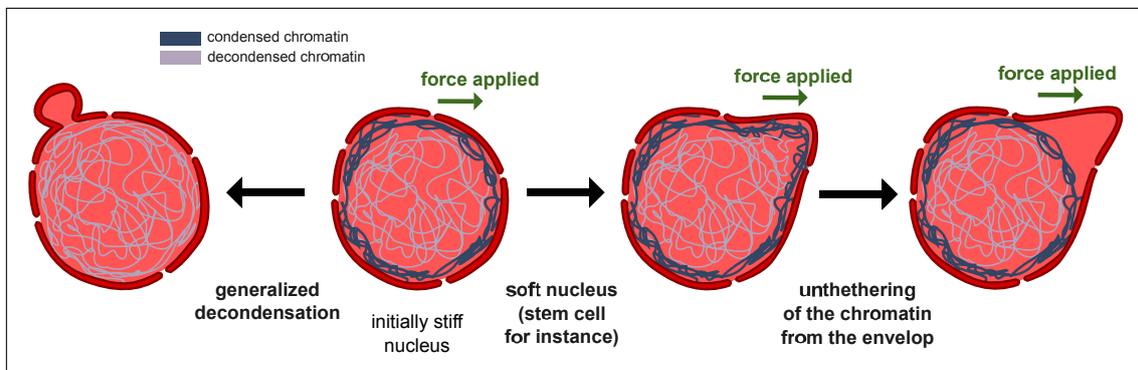


Figure 1.9 – Chromatin tethering contributes to nuclear mechanical properties depending on its condensation state. Decondensation of chromatin induces nuclear blebbing and indicates that chromatin participates to nuclear envelope integrity. Moreover chromatin follows envelope movements and a stiff nucleus buffers the force transmitted to the chromatin.

1 Chromatin contribution to nuclear sturdiness

Chromatin even though organized is still a mass with its own physical properties. As intuitively expected, impairing chromatin interaction with the envelope leads to a softer nucleus. It shows that chromatin tethering puts up resistance to nuclear deformation [Schreiner *et al.* 2015] (figure 1.9). It follows nuclear envelope movements and as such contributes to the nucleus mechanical properties. Due to the fact that heterochromatin and euchromatin have different physical properties, the first one being denser than the second, nuclear mechanics can be modified by global chromatin condensation state: the more condensed the stiffer the nucleus is [Furusawa *et al.* 2015] [Shimamoto *et al.* 2017]. What happens on the contrary, when chromatin is decondensed, is that the nucleus gets

an abnormal shape identified by nuclear blebbing. It can even go to formation of micronuclei [Stephens *et al.* 2018] (figure 1.9). Similar results were obtained when modulating formation of chromocenters, which are aggregates of heterochromatin. Their formation participates to nuclear integrity: if chromocenter formation in blocked nuclei forms blebs similar to what has been observed with global decondensation [Stephens *et al.* 2018]. Leakage from the nucleoplasm to the cytoplasm can be observed as well. These results illustrate that chromatin can influence nuclear shape and that its compaction state maintain the integrity of the nuclear envelope.

Chromatin response to mechanical stress is also adaptive. Cyclic forces applied to the nucleus affect condensation state of the chromatin that will remain over time. In other words, chromatin state reflects mechanical events the cell has been subjected to and act as a memory of the cell's physical history [Haase *et al.* 2016]. Going in the same direction, it has been shown that nuclear mechanical response in isolated cells depends on its previous mechanical environment. Cells cultivated on a substrate favouring cell-matrix contact or on the contrary on a substrate favouring cell-cell contact display different morphologies, thus forces experienced by the nucleus will be different and will induce different chromatin organization [Balikov *et al.* 2017]. All this data brings back and expands the concept of the niche, adding to the short chemical range signalling the physical probing of the environment. The relationship between chromatin state and mechanical properties of the nucleus resonate very well with a mechanosensing mechanism. Going further, studies show that lamins are at least as much important as chromatin for nuclear mechanics integrity.

2 Lamins contribution to nuclear sturdiness

There are three isoforms of lamins: A, B and C [Gruenbaum & Medalia 2015]. Lamins A and C are usually grouped together due to their role in nuclear stiffness, whereas lamin B is responsible for nuclear integrity and elastic response [Lammerding *et al.* 2006].

Abnormalities in lamin content have been observed in multiple diseases grouped under the term laminopathies. The most striking one is progeria. Patients having this mutation exhibit a premature senescence phenotypes starting the second year of life, with normal intellectual capacities. When the nuclei of the cells of these patients are observed under the microscope, they present a completely irregular shape [Davidson & Lammerding 2014].

Correlated to this abnormal nuclear shape, these cells have an increased number of DNA damage as well as abnormal chromatin organization. Strikingly expression of the lamin mutant responsible for progeria (progerin) in human mesenchymal stem cells induces a cell identity change and reduce their potency similarly to what is observed during ageing [Scaffidi & Misteli 2008]. On top of that, it alters chromatin organization [Briand & Collas 2018]. It strongly suggests that this truncated form of lamin, because not able to preserve proper nuclear shape, disorganizes chromatin provoking stem potency loss.

Surprisingly lamins are not the universal constituents of the lamina. In plants or unicellular organism other proteins assume this role. In plants they are called nuclear matrix constituent proteins (NMCP) and in trypanosoma, NUP-1. These proteins are analogue (and not homologue) as they have very different sequence but similar tertiary structure. They localize at the nuclear periphery and seem to have similar function regarding nuclear integrity. For instance NUP-1 deletion leads to nuclear deformation and even loss of structure integrity [DuBois *et al.* 2012] similar to the phenotype induced by loss of function of lamins in progeria. Why lamina components have followed very distinct evolutionary pathways is not determined yet but their analogous relationship strongly strengthens the role of nuclear mechanics for gene expression and cell identity maintenance.

Several laminopathies exists and are linked to particular mutations in the lamins gene leading to organ specific phenotypes. Similarly lamin B receptor (LBR) a membranous protein located on the inner nuclear membrane (INM) participate to the anchorage of the lamina to the nuclear envelope and which is needed for proper neutrophils function [Gaines *et al.* 2008].

Experiments on isolated nuclei highlighted the fact that the nucleus can respond by itself to mechanical cues via lamin A/C recruitment [Guilluy & Burridge 2015]. This result could be an explanation for the chromatin dependent stiffening of the nucleus. While the nucleus is subjected to forces, lamins A/C are recruited to the envelope, increasing its stiffness and increasing interaction probability with chromatin leading to compaction. Hematopoietic stem cells differentiation can be directed towards a specific lineage solely on modulating lamin A expression, high level of lamin A leading to erythropoiesis and the opposite towards megakaryogenesis [Shin *et al.* 2013]. This observation raises the question

of the interplay between nuclear mechanics and chromatin, how one can influence the other and how this interplay can be involved in the control of differentiation.

Interestingly, during differentiation, lamin content changes: lamin A/C increases compared to lamin B [Shin *et al.* 2014]. As stated previously this is indicative of a stiffening of the nucleus [Lammerding *et al.* 2006] showing that stem cells are in a particular state allowing them to respond to external stimuli, and that differentiation modify this ability [Heo *et al.* 2016]. It means gene expression will afterwards, be differentially affected by forces (figure 1.10).

3 Evolution of nuclear mechanical properties during differentiation

Embryonic stem cells entering differentiation exhibit auxetic nuclei which means pulling them expands them in every direction instead of extending it only in the direction of the pulling force [Pagliara *et al.* 2014]. Similarly compressing them in a given direction compact them instead of squashing them. This is thought to be due to differences in chromatin condensation state happening during differentiation, but how chromatin can become auxetic is still not understood. It can have non negligible effects, as auxetic nuclei will respond differently to physical stresses. The same force will in the auxetic case increase available volume for protein-chromatin interaction and in the non auxetic case the volume will be decreased increasing nuclear density. These two nuclei will respond in two different ways to an identical mechanical stimuli [Pagliara *et al.* 2014].

4 Stem cell heterogeneity participates to differential mechanical response

Heterogeneity of expression profiles found in stem cells could reflect heterogeneous mechanical properties found in embryonic stem cells. Some have a soft nuclei that will adapt to the cellular shape, others will have stiff nuclei that will remain spherical even upon constraints. Despite this heterogeneity these two populations still have embryonic stem cells capacities. The stiff nuclei have an increased lamin A content as well as increased chromatin condensation marks and could explain the little deformability. As suspected the difference in nuclear mechanical properties affects chromatin dynamics [Talwar *et al.* 2014]. It was also shown that the stiff phenotype correlates with myocardin-related transcription fac-

tor A (MRTF-A) localization in the nucleus [Talwar *et al.* 2014] and suggests that nuclear mechanical properties can be linked to a differentiation potential.

In the light of those informations it seems quite hard to determine who comes first. Is the nucleus stiffer because the cell has already started differentiation, even just a little ? Or is it because it has been subjected to strains that lamin content has been changed and differentiation is now biased towards another path. If we come back to the heterogeneous stem cell population hypothesis it might be easier to link these events. What heterogeneity of stem cells means is that they have different expression profiles although they have the stem cell potency. Stochasticity of gene expression could explain this phenomenon and might be necessary to access different stem states. For instance, in a very different system, at the 32-cells-stage of the mouse embryo, cells' genes expression is not correlated to their position. This allows the cell to express for instance a receptor, at a given time and be sensitive to an external stimuli. It will lead to a signalling pathway activation, acquisition of a new identity that in turn will influence the one of its neighbours [Simon *et al.* 2018]. What it means is that stochastic gene expression (SGE) allows the cell to access numerous states (like a stiff nucleus) that will allow it to respond in a specific manner to external stimuli. What can initially thought to be inevitable noise is a way to expand available states to enter differentiation.

5 Lamin response to mechanical stress

We have seen that lamins can impact chromatin organization. Next question is about what can impact lamins, quantitatively or qualitatively. It was shown that changing nuclear shape by the intermediate of cell shape, provokes a change in lamin phosphorylation and turnover [Swift *et al.* 2013] [Buxboim *et al.* 2014]. Consequences could be that upon bearing loads lamin interaction with chromatin would be modified. It strongly suggests that when deformed, dynamics inside the nucleus are changed and heterochromatin could be freed from the envelope for further activation.

Altogether these experiments shed light on the mechanisms of substrate stiffness dependent MSC differentiation [McBeath *et al.* 2004]. Indeed, by modulating cell shape, thus nuclear shape, via substrate stiffness, it could possible to modulate lamin organization. Then chromatin would reorganize and it would eventually modulate gene expression in-

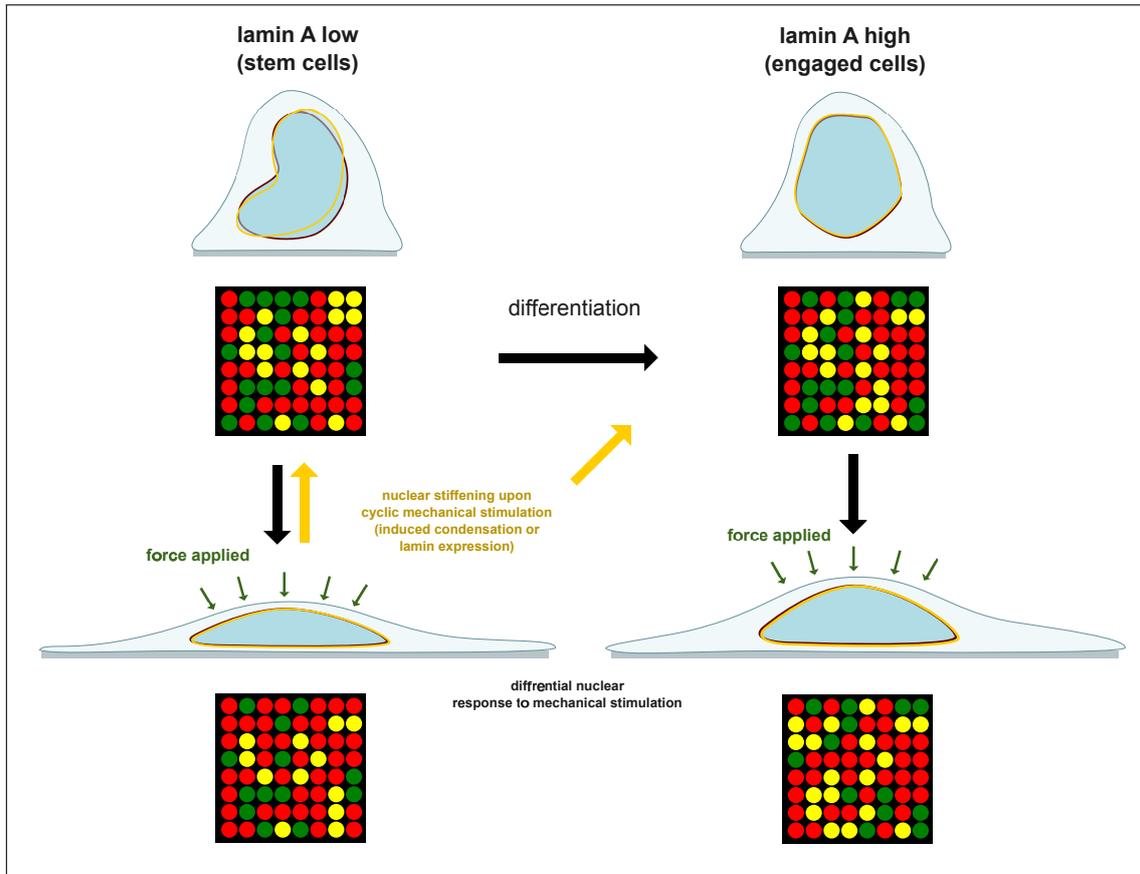


Figure 1.10 – Nucleus stiffening upon cyclic stimulation or by lamin acquisition upon differentiation changes its mechanical properties, therefore changing nuclear response to forces. Soft nuclei will deform and fluctuate over time and for an equivalent compression forces will be easily transmitted to the chromatin impacting its organisation.

involved during differentiation. This is possible because the nucleus is linked to cytoplasmic components like the cytoskeleton which governs nuclear shape.

6 Lamins interaction with the cytoskeleton

Emerin is a membranous protein, anchoring lamin to the envelope. Emerin is not as important as lamins for nucleus' mechanical properties but is critically involved in mechanotransduction [Lammerding *et al.* 2005] [Gaines *et al.* 2008] because it links the lamina to proteins of the outer nuclear envelope that will interact with the cytoskeleton: the linker of nucleoskeleton and cytoskeleton (LINC) complex [Osmanagic-Myers *et al.* 2015].

The LINC complex is composed of proteins containing the SUN domain (SUN protein in mammals) and KASH domain (nesprin in mammals) [Graham & Burrridge 2016]. These

two proteins form a complex that goes from the nucleoplasm to the cytoplasm, SUN being on the inner side interacting with the lamina, nesprin attaching to SUN in the intermembranous space and pointing out in the cytoplasm, interacting with the cytoskeleton (figure 1.11). This complex is the intermediary between the outside and the inside of the nucleus, almost all mechanotransduction events go through it. It is via nesprin signalling that isolated nuclei are able to regulate their lamin content [Guilluy & Burridge 2015]. It is also thanks to the LINC complex that mesenchymal stem cells are able to integrate substrate stiffness into the differentiation process [Alam *et al.* 2016]. LINC complex defects impair correct mechanotransduction and are causes of diseases [P. Isermann 2013]. The relationship between the nucleus and the cytoskeleton has to be considered to be able to understand how mechanical events are integrated in the chromatin.

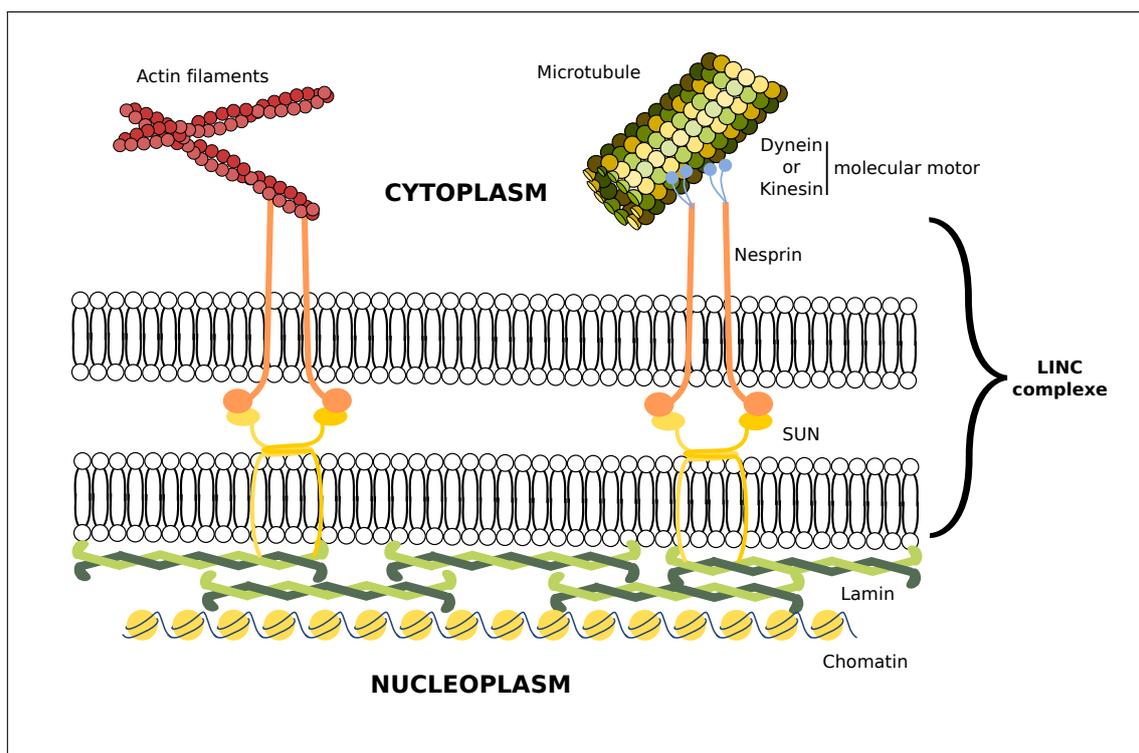


Figure 1.11 – Chromatin is linked to the cytoplasm components via the LINC complex. It consists of a SUN protein domain located in the inner nuclear membrane that binds with lamin, itself binding with chromatin. In the inter membrane space SUN domain protein binds a KASH domain protein, which is a transmembranous protein in the outer nuclear membrane. KASH domain protein can bind with actin or molecular motors associated to microtubules. Doing so, forces generated by the cytoskeleton are transmitted to the nucleus.

3 Current Issues

Although through decades the description of chromatin organization was greatly improved, less is known about how mechanical information is transmitted to the chromatin, in other words, what happens to the chromatin when the envelope is put under constraints. Due to the overall architecture of the chromatin-lamin-LINC complex ensemble, forces applied on the nucleus can be transmitted to the chromatin and potentially impact its organization. Indeed, when the nucleus is stretched, the force is directly transmitted to the chromatin [Miroshnikova *et al.* 2017] [Jacobson *et al.* 2016]. This puts the chromatin filament under tension and can in the short term affect its dynamics and its expression via rapid specific promoter activation. This activation is likely provoked but not only by force-induced decondensation. When inducing shear forces on the nucleus it was shown, by measuring distance between two loci via FISH, that these forces have direct impact on chromatin decompaction [Tajik *et al.* 2016]. Strikingly, correlating with decompaction, transcription was also rapidly activated showing that force dependant gene expression is possible. On the long term, forces have a different effect. It induces a switch of chromatin marks from H3K9me3 to H3K9me27, from strongly repressed to fluctuating repression of genes. Even though a repression mark, H3K9me27 is more flexible and participates to the appearance of new gene expression control points [Miroshnikova *et al.* 2017].

Forces to which the nucleus is subjected also have consequences on the integrity of the nuclear envelope. Studies show that during migration through pores the nuclear envelope is ruptured [Raab *et al.* 2013] [Denais *et al.* 2016], actin cytoskeleton being involved in this phenomenon [Thiam *et al.* 2016]. Leakage in the nucleus inevitably leads to cytoplasmic components presence in the nucleus that could have lots a consequences on DNA integrity. In the stem cell case these kind of phenomenon could be of great impact on differentiation.

Mechanics of the nucleus have been shown to be important in the differentiation process, mostly as a force transmitter. The way it happens, how nuclear deformation can affect global chromatin organization are just being described nowadays. But previous examples of force mediated nuclear structure or chromatin changes were obtained using forces generated by the experimenter which do not reflect physiological conditions of differentiation.

However cells are able to generate forces themselves via the cytoskeleton. Thus it should be possible to reproduce similar results modulating solely cellular organization.

III Nucleus and cytoskeleton

The cytoskeleton is composed of two types of very dynamic filaments (actin and microtubules) and one less dynamic (intermediate filaments). Here only actin and microtubules will be discussed as the role of intermediate filaments in force generation still remains unclear.

1 Actin: the main force generator in adherent cells

1 Structure and organization

Actin forms polarized filaments (5nm) due to the polarity of its forming unit, monomeric globular actin (g-actin). It has asymmetric affinity sites for other actin monomers and this is responsible for the polarized characteristic of the filaments. The polymerizing front is called the plus-end (or barbed-end). Polymerization is favoured when g-actin is bound to ATP. When polymerized, ATP will be hydrolysed into ADP, leading to a change of conformation, destabilizing the filament, favouring depolymerizing front at the opposite end of the polymerization one. Finally, ADP of the released g-actin will be exchanged for ATP, ending the cycle. This constant directional polymerization/depolymerization makes the filament virtually move in a given direction, it is the threadmilling effect. In the cell, actin filaments are quite stable because of the very slow depolymerization rate compared to the polymerization one. To regulate the polymerization and arrangement of all those filaments many proteins are expressed by the cell (crosslinkers, severing proteins, capping protein which stops growth, nucleator...) to control fibers' size, quantity etc. [Blanchoin *et al.* 2014].

Actin filaments are thus dynamic, used to build a dynamic network and can be rearranged to switch quite fast between different organizations. As already stated, structures created by actin filaments make a framework giving the cell its shape. This framework consists of two main structures among secondary others (figure 1.12).

- Branched network. A proteic complex (ARP2/3) will bind a filament and nucleate a new filament at a specific angle. This creates at a large scale a branched structure similar to a tree, covering a large area. This network is found in the lamellipodia, at

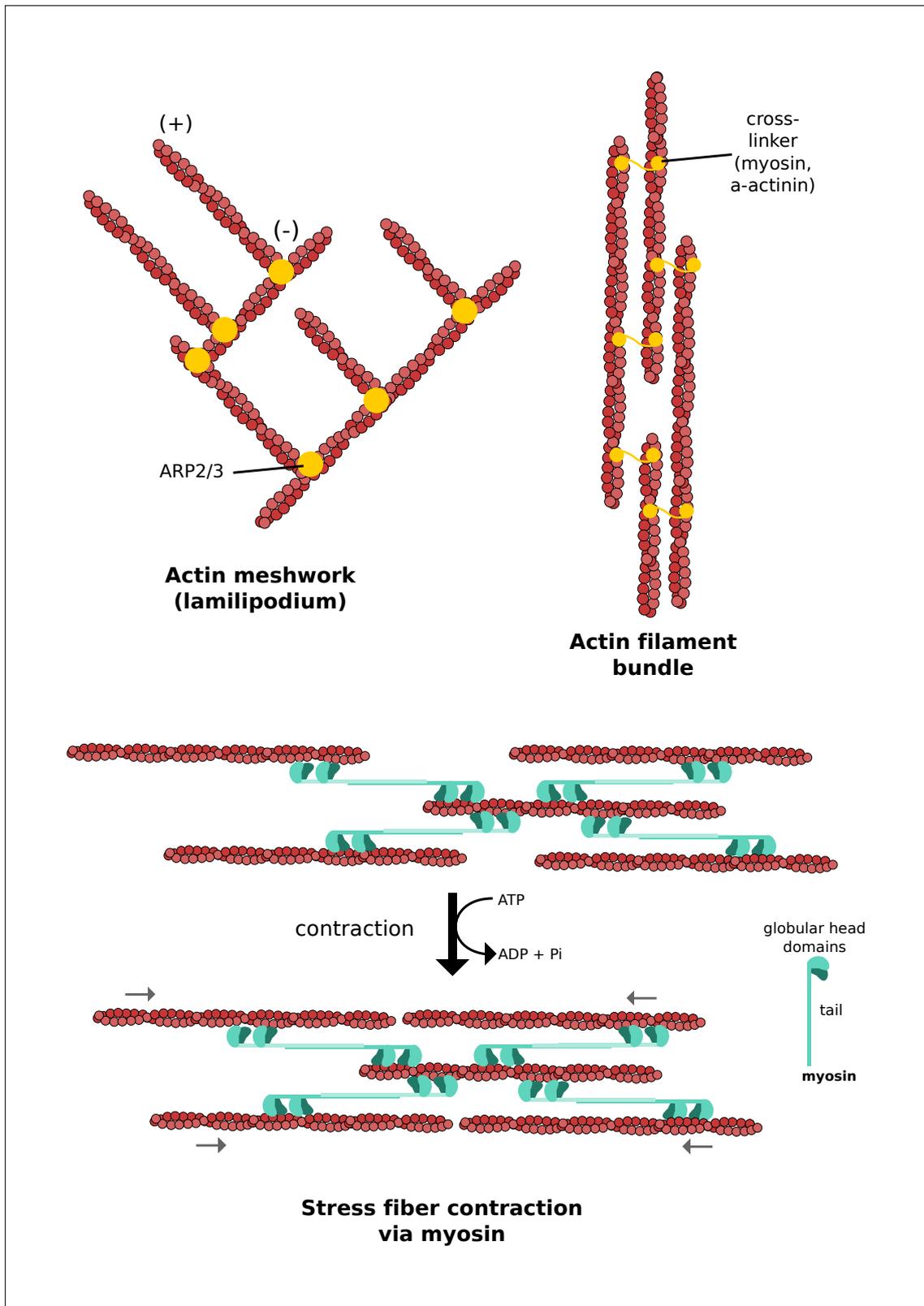


Figure 1.12 – Two main actin organization can be found in adherent cells. The branched network is able to cover large surface for the cell to extend and scan its environment. This structure is found at the leading edge of the cell and propels it. Stress fibers are contractile bundles of actin fibers that will participate to the cell contractility.

the leading edge of migrating and spreading cells. It allows the cell, in combination with stress fibers, to extend its membrane and migrate by pulling it on the substrate. The cell crawls.

- Actin bundles. Made of the association of multiple actin filaments linked together either in a parallel or anti-parallel fashion by a passive (alpha-actinin for instance) or active crosslinker (non-muscle myosin). In the latter case the bundle is called a stress fiber. Myosin is composed of a head and a tail, and form anti parallel bundles binding actin by the heads (figure 1.12). With consumption of ATP, myosin is able to walk on the actin filaments thus increasing the overlap between the two, it is contraction (figure 1.12).

When a cell is plated on a surface, it goes from a spherical shape to a spread one. It generates proteic complexes, focal adhesions, which anchor themselves on the surface by the intermediate of proteins called integrins. Focal adhesions are stabilized when forces are applied on them, those forces are generated by the actin fibers contraction. On a soft substrate, focal adhesions can't be efficiently stabilized due to the poor force transmission to the substrate and prevents cell spreading. On the contrary on a stiff substrate focal adhesions are easily stabilized because of the strong substrate reaction force. The cell is able to spread, enters a positive feedback loop increasing the number of focal adhesions. At the end of the day, the cell reduces its height. Fibers going on top of the nucleus will compress it and deform it [Ramdas & Shivashankar 2015] (figure 1.13).

2 Stress fibers generate forces on the nucleus

Actin organization around the nucleus is involved in its mechanical response. Actin stress fibers going over the nucleus form a polarized array: they are aligned with each other and do not overlap. It means that when contracting they generate anisotropical forces. Thus, when the cell contracts, the nucleus will stretch more in one direction than in another [Haase *et al.* 2016]. Going further, it means that chromatin will sense different forces depending on its position in the nucleus.

Another example illustrating the ability of actin to apply forces on the nucleus is during migration. During wound healing assay, to close the wound, cells at the edge start to move and proliferate to close it. During these events, the centrosome goes between the nucleus and the leading front of the cell, coming from behind the nucleus. Actin fibers, perpendicular to the migrating direction coming from the leading edge, from the lamellipodium, move towards the nucleus and go over it. Interestingly, SUN-2 and Nesprin-2 colocalize with the fibers and form striations on the nucleus. This is indicative of actin fibers anchoring at the nucleus via the LINC complex. When actin interaction with nuclear SUN-2 is altered the repositioning of the centrosome does not happen [Gant Luxton *et al.* 2011]. This result shows that actin is able to generate forces on the nucleus and that the LINC complex is absolutely necessary for it. Surprisingly, SUN isoform SUN-1 does not colocalize with actin and do not form any striation. To sum up, the flow of actin is able to push the nucleus back, allowing the centrosome to go in front of it for proper migration to begin [Lee *et al.* 2007] [Folker *et al.* 2011]. This set of experiments also shows that expression of specific isoforms of the LINC complex can be controlled for specific mechanisms to occur.

In the same way external forces applied to the nucleus can modulate chromatin organization, actin fibers and their ability to bind LINC complex also should, physiologically, impact chromatin positioning, and thus gene expression.

3 Consequences of actin-driven forces on nuclear architecture

Actin network constitutively apply compressive forces on the nucleus. Nucleus squeezing has consequences on nuclear architecture, among which it creates a lamin distribution asymmetry between the top and the bottom of the nucleus [Kim & Wirtz 2015]. This result is supported by the fact that lamin conformation is load dependant and can change its conformation [Ihalainen *et al.* 2015]. What it means is that lamins at the top and at the bottom of the nucleus are distinguishable and are susceptible to interact in different manners with chromatin. It also means that actin generated forces on the nucleus can impact lamina. So modulating actin cytoskeleton seems a reasonable way to modulate interactions between nuclear envelope and chromatin that might in turn impact gene expression.

It was shown that modulating contractility or actin organization in spread cells, can impact nuclear architecture. One way of doing so is to put cells on a substrate of a defined

shape and size. It can be achieved by micro patterning of proteins on a glass substrate. One of the technique used to obtain micro patterns consists of coating a glass surface with hydrophobic, cell repellent, molecules. Then remove the cell repellent with deep UV illumination through a mask containing micron scaled shapes. The repellent is removed where the shapes are and are then coated with adhesion proteins such as fibronectin. Micro patterning approach is similar to modulating substrate stiffness as limiting adhesion surface recreates intracellular architecture of cells on soft substrate and in both cases spreading is reduced and cells can't form stress fibers over the nucleus. On large patterns, stress fibers can be found on top of nuclei [Li *et al.* 2014]. It ends up that micropatterning can be a tool to tune gene expression via its impact on nuclear shape, with constant biochemical environment [Gupta *et al.* 2012]. Another method is to stretch the substrate the cells are on.

Increasing cell contractility has been shown to induce chromatin methylation (H3K27me3) indicative of gene repression [Le *et al.* 2016]. Ultimately it leads to protein synthesis modification [Thomas *et al.* 2002]. On the contrary when depolymerized or myosin activity is blocked, the nucleus relaxes and appears swollen [Ramdas & Shivashankar 2015]. Seemingly, reducing tension on the nucleus by micropatterning makes the envelope prone to fluctuations responsible for chromatin movements, especially telomeres [Makhija *et al.* 2015]. Interestingly telomeres located close to the nuclear envelope follow more the envelope than the ones present at the nuclear interior. It strongly supports the interplay between nuclear and chromatin dynamics. What has also been observed is that on constrained nucleus, lamin A is more expressed than in non constrained. This has to be connected to experiments on isolated nuclei where cyclic force application induces lamin A recruitment. Here, it is demonstrated that in cells, nuclear compression by actin filaments cause lamin A recruitment that will in turn impact chromatin dynamics [Makhija *et al.* 2015].

To sum up all this data, it ends up that actin is forming a kind of cage around the nucleus constitutively applying forces on it. Removing actin releases pressure, the nucleus swells back. In the first case the nucleus is trapped and is kind of fixed but when tension is released it starts to fluctuate. These fluctuations are transmitted to the chromatin and modulate its dynamics [Chu *et al.* 2017]. Lamin A seem to be the crucial intermediate

between cytoskeleton and chromatin organization either by its ability to interact with chromatin or to stiffen the nucleus. Similar morphological transitions can be observed in other models, including stem cells.

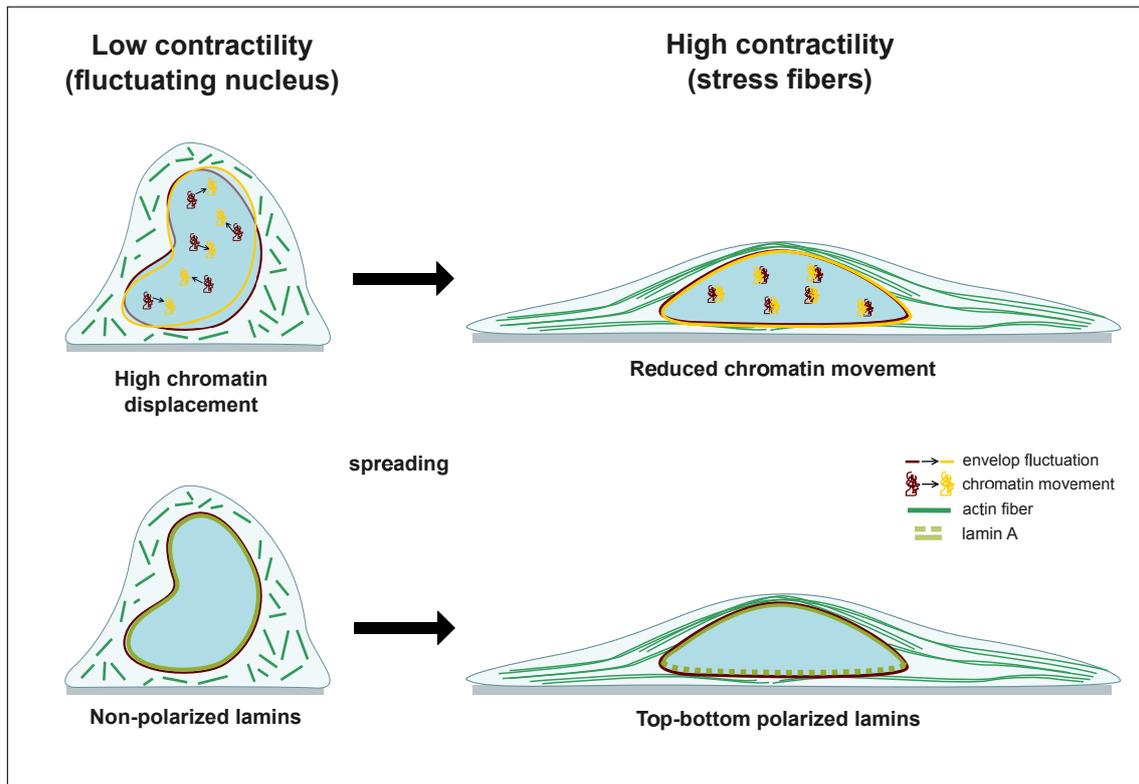


Figure 1.13 – During cell spreading actin starts to form stress fibers going on top the nucleus. When contracting these fibers exert a compressive force on the nucleus.

4 Actin architecture transition in stem cells

Human pluripotent stem cells (hPSCs) change their actin architecture when they differentiate. Initially they form colonies which have very specific organization. They are not formed of spread cells but by weakly adherent cells. Cells at the edge of the colony generate actin cables specifically on the edge side of the colony. They end up making a fence of actin around the colony (figure 1.14 left). This structure, because confining the cells prevents formation of actin stress fibers in the cell which means the nucleus is not compressed, just like when the spreading surface is reduced via micro patterns. When the differentiation is triggered this fence is broken and the cells start to spread thus compressing the nucleus (figure 1.14 right). Gene expression changes are associated to this morphology transition

and especially stem cell genes [Närvä *et al.* 2017]. Changes in chromatin organization can be expected but have not been reported yet. What is important in this set of data is that provoking this actin reorganization by perturbing actin fence integrity leads to the spreading of the cells [Närvä *et al.* 2017]. This spreading is associated with the same gene expression modification than during differentiation, although of a lower intensity. Thus, without inducing differentiation it is possible to affect stemness solely by changing cellular morphology [Närvä *et al.* 2017]. Nuclear shape is sufficient to alter gene expression.

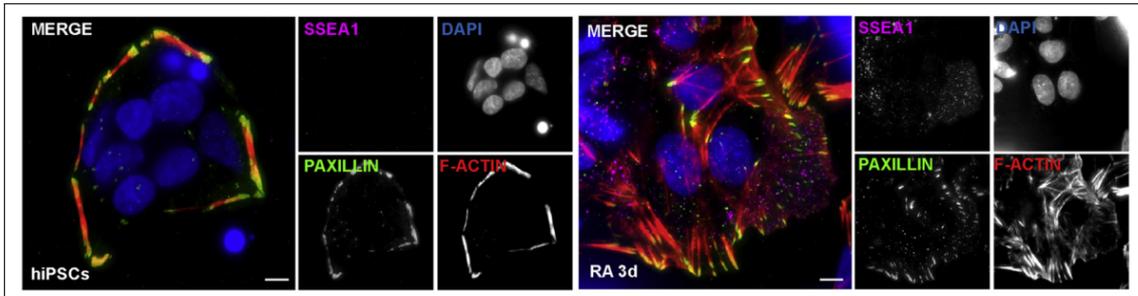


Figure 1.14 – Human pluripotent stem cells form colonies with specific actin architecture. When differentiating their morphology becomes fibroblast-like (mature adherent cells). Paxillin are used as a marker of focal adhesion and SSEA-1 as a marker of differentiation [Närvä *et al.* 2017].

The nucleus is a mechanoresponsive organelle, and by modulating actin, the major force generator in adherent cells, it is possible to affect, at least partially, chromatin organization and gene expression. All of these elements shed light on the mechanisms of mechanotransduction and on the importance of nuclear interaction with the cytoskeleton during differentiation [Uzer *et al.* 2016]. Here, only actin was described, but there is another dynamical cytoskeleton element in the cell: microtubules.

2 Microtubules: another way to constraint the nucleus

1 Structure and organization

Microtubules are organized in a very different manner than the actin filaments. First of all, they are larger (25nm) and formed by the polymerization of a tubulin dimer (beta-tubulin + alpha-tubulin) (figure 1.15). The building block dimer binds GTP, hydrolysing it into GDP when integrated into the polymerizing front. Here again difference in polymerization speed at plus-end and depolymerization speed at the minus-end lead to a treadmilling effect. In mammalian cells, most of the microtubules are generated from a single struc-

ture: the centrosome (figure 1.15). It is a structure composed of two centrioles, also made of microtubules, interacting with proteins of the pericentriolar material (gamma-tubulin, pericentrin...). Altogether, they form the microtubules organizing center (MTOC), a small non membranous organelle nucleating microtubules. Because growth always starts at the centrosome, microtubules plus-end is always located at the periphery of the cell. Microtubules are inherently unstable, as opposed to actin filaments. They grow, depolymerize abruptly (catastrophe event at the plus-end), and then regrow (rescue event) and so on. This characteristic was named dynamic instability. Microtubules can also be crosslinked, either by passive or active crosslinkers, and are responsible for formation of larger structures called bundles.

As for actin, active crosslinkers are able to make microtubules structures generate forces: kinesin and dynein, both composed like myosin of a head and a tail. Dynein and kinesin differ by their directionality, the first one goes towards the plus-end, the second one towards the minus-end (figure 1.15). When microtubules are crosslinked together by molecular motors the resulting sliding can generate forces, either contraction (in the case of dynein) or expansion (in the case of kinesin) [Lu *et al.* 2015]. Sliding can generate forces on the cell membrane and deform it as it could be observed in megakaryocytes. Megakaryocytes are huge cells in the bone marrow that generate platelets by tearing apart its membrane through sinusoids endothelial cells. These protrusions will be torn by the flow induced shear stress and the resulting anucleated cell is the platelet. For this process to occur microtubules and dynein motors are needed. The mechanism has not been described yet [Bender *et al.* 2015].

2 Tracks to position structures in the cytoplasm

Motors' tail can bind components of the cytoplasm and then position them at a specific place. The classic example is the enterocyte which secretory vesicles are only transported towards the lumen side of the cell and contributes to the polarization of the cell.

Dynein and kinesin can be anchored at the cytoplasmic membrane by nuclear mitotic apparatus complex (NuMA) [Okumura *et al.* 2018] and participate to spindle positioning during mitosis [Laan *et al.* 2012a] [Laan *et al.* 2012b] [Tanimoto *et al.* 2016]: chromosomes will be gathered at the center of the spindle in order to be segregated in the daughter cells.

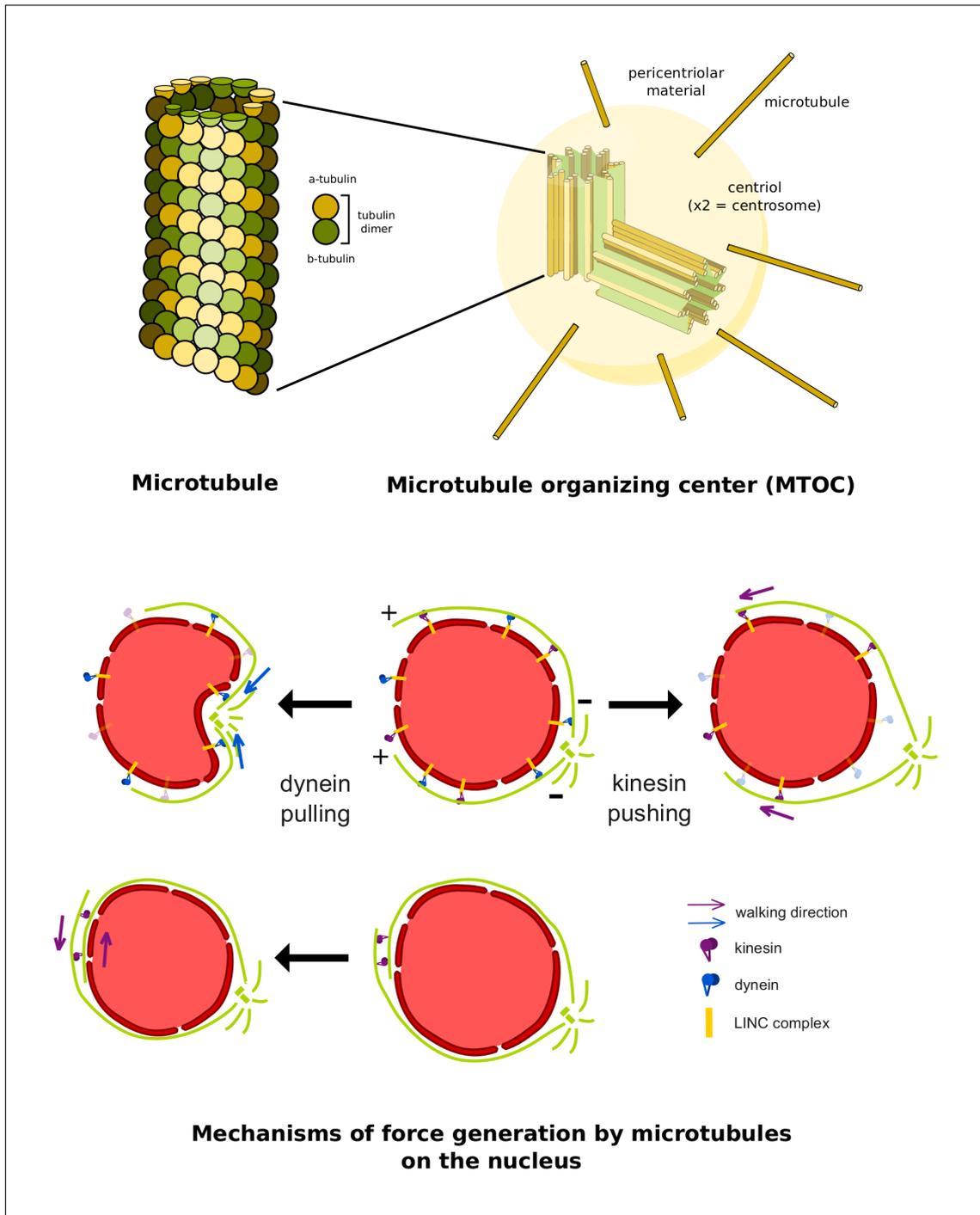


Figure 1.15 – Microtubules are 25nm diameter tubes formed by polymerization of a tubulin dimer (alpha+beta). Microtubules are nucleated from the MTOC forming an aster. In mammals MTOC is formed of two centriols (centrosome) themselves formed by a 9 fold symmetry microtubules triplets. Microtubules can interact with molecular motors (dynein and kinesin). These motors can be anchored at the LINC complex level and generate forces on the nucleus. Kinesin will move away the centrosome and the nucleus whereas dynein will bring them together. Dynein can also induce microtubule sliding responsible for compressive forces on the nucleus.

This process involves microtubules depolymerization which generate pulling forces on the chromosomes.

Molecular motors are also critically involved in nuclear movements. Motors' tail can bind neprins from the LINC complex and by doing so, dyneins walking towards the minus-end are capable of pulling the nucleus to the centrosome keeping them close together. On the contrary kinesins walking toward the plus end will push the centrosome and the nucleus apart (figure 1.15). Balance between kinesins and dyneins activity avoid dissociation of the two organelles in interphasic cells [Akhmanova 2011].

In some cases like migration, nucleus has to follow the cell movements not to act as a weight for cell displacement [Roux *et al.* 2009] [Fridolfsson & Starr 2010] [Bone *et al.* 2016]. Nuclear oscillations induced by kinesin and dynein are needed for proper nuclear positioning during migration direction changes [Akhmanova 2011]. If taken to the limit, motors activity at the nuclear level could remove the nucleus from the cell. For instance, during erythroblast maturation, a process during which the erythroblast cell is enucleated to generate the functional red blood cell, dynein activity is necessary for enucleation although the mechanism still remains unclear [Kobayashi *et al.* 2016]. The last hypothesis potentially leading to nuclear deformation is if microtubules growing from the centrosome surround the nucleus and then overlap. Sliding by dynein would compress the nucleus like a vice (figure 1.15).

3 Another way to deform the nucleus

During interphase, microtubules induced forces seem to participate to nuclear shape although in a lower range than those generated by actin filaments [Ramdas & Shivashankar 2015]. Nevertheless, it is possible to observe strong deformations under certain conditions. During polymerization of microtubules from the MTOC, after drug treatment depolymerization, the centrosome tends to form an invagination in his vicinity, possibly induced by dynein bound to LINC complexes pulling on newly formed microtubules [Gerlitz *et al.* 2013] (figure 1.15). Another way to illustrate the capacity of the microtubules to deform the nucleus is by playing with the lamins content. Upon lamin A depletion, interphasic cells display a large invagination that correlates with centrosome position [Tariq *et al.* 2017] that disappear when microtubules are depolymerized. Same

goes when the interaction between the microtubules and the nucleus is increased by over expression the LINC complex [Donahue *et al.* 2016].

On the one hand these results suggest that during interphase in adherent cells, microtubules are capable to generate weak forces on the nucleus. On the other hand there are physiological situations where microtubules are capable of generating much higher forces. For instance, for mitosis to occur, nuclear envelope breakdown (NEB) is needed. Microtubules, generated from both duplicated and original centrosomes, grow against the envelope making small invaginations in the nuclear envelope. Then dynein walking towards microtubules minus-ends and bound to the LINC complex tear the envelope down [Gerlich *et al.* 2002]. When mitosis is over, microtubules serve as tracks to reform the nucleus close to the centrosome highlighting a strong bond between those two structures [Xue & Funabiki 2016].

When bundled, microtubules are able to generate higher forces. Bundles formation can be induced for example by over expression of passive crosslinkers (like the TAU protein). In this configuration they can drastically deform the nucleus [Monroy-Ramirez *et al.* 2013]. Similarly large bundles of microtubules forming a structure called the *manchette* (figure 1.16), are known to induce large scale deformation of the nucleus during the sperm cell maturation. This structure is needed to compress the nucleus and compact the chromatin in order give the sperm head its final functional shape [Russell *et al.* 1991]. In this system, integrity of the LINC complex is needed otherwise the *manchette* is not able to generate the required forces on the nucleus [Pasch *et al.* 2015].

Finally, in tissues, nuclear envelope breakage was observed during interphase. To repair these ruptures lamins are recruited but interestingly dynein act oppositely to the closing process, as their removal facilitate the repair [Penfield *et al.* 2017].

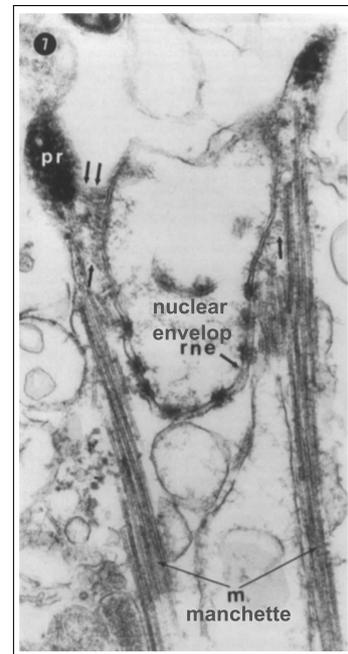


Figure 1.16 – Large microtubules bundles can be observed surrounding sperm cell nucleus and deforming it [Russell *et al.* 1991].

These examples show that microtubules are able to generate forces on the nucleus strong enough to deform it just as actin does. It thus seems reasonable to infer that microtubules can participate to chromatin organization.

4 Consequences of microtubules-driven forces on chromatin

If little is known about microtubules influence on interphase nucleus, even less is known about the role of microtubules in chromatin organization nor in stem cells differentiation. If we consider the MTOC evolution in eukaryotes it might not be surprising that this structure and the microtubules associated can impact chromatin organization.

Centriols form the centrosome but are also at the basis of the flagellum (basal body), a long protrusion formed by microtubules and allowing cell motility. It can be observed in euglenids or plankton for instance. Some eukaryote organisms like yeast and dictyostelium have acentriolar MTOCs, respectively called spindle pole body (SPB) and nucleus associated body (NAB), and interestingly they are also deprived of flagellum. The SPB is embedded during the whole cell cycle whereas the NAB is strongly attached to the nucleus but can dissociate during interphase. What is thought is that the acentriolar nature of the MTOCs is a secondary evolutionary loss, due to non flagellar motility and that before nucleus appearance centriol from the flagella would have duplicated to serve as a clustering agent for DNA which started to attach to the endomembrane system of the organism [Gräf *et al.* 2015]. In the end, the loss of a flagellum based motility in yeast would have made the centriolar nature of the centrosome irrelevant and the MTOC remained exclusively attached to the nucleus mostly for chromatin organization. SPB and NAB are indeed centrometric clustering region and strongly suggests that due to their evolutionary relationship with centriols that the centriolar centrosome of mammalian cells is still able to interact strongly with chromatin.

In fission yeast *S. pombe*, the region of the nuclear envelope associating with the SPB contains specific KASH proteins isoforms [King *et al.* 2008]. Interestingly specific heterochromatin regions, centromeres, are directly bound to these KASH proteins and thus provide an indirect interface via the SPB and the microtubules. Any forces coming from the microtubules will be transferred to the chromatin [King *et al.* 2008]. Similarly specific MTOCs called telocentrosomes are formed at the interface of telomeric regions and the

nuclear envelope. They are formed by recruitment of LINC complex components and require centrosomal activity associated proteins. Then microtubules generated from these MTOCs interact with each other via dynein, bringing together both telomeres at the SPB. Homologous chromosome pairing for meiosis recombination can occur [Yoshida *et al.* 2013] [Katsumata *et al.* 2017]. These examples show how chromatin and microtubules network can be intertwined as specific chromatin regions can be moved around the nuclear envelope by interacting indirectly with microtubules.

To come back to multicellular organisms, in drosophila meiotic cells, nuclear movement are also required for proper centromeric region clustering and depend on a microtubules based mechanism. Dyneins drive rotation of the nucleus and increase centromeres encounter for pairing. If blocked, the time needed for this process to happen is increased [Christophorou *et al.* 2015]. During interphase, microtubules can also induce chromatin movement as demonstrated during DNA double strand break (DSB). When breakages occur, increased mobility of surrounding chromatin is observed. It is dependant of the LINC complex integrity and microtubules dynamics [Lottersberger *et al.* 2015]. These movements increase the chromatin mobility, just like during meiosis pairing, to favour encounter that will reconnect the free ends. It is particularly important as after breakage the distance between the two free ends can be dramatically increased [Lottersberger *et al.* 2015]. This study shows that microtubules have the ability to organize chromatin during certain events and suggests that microtubules dynamics can impact chromatin organization during differentiation.

5 Potential actors for gene expression control

Drosophila embryo cellularization is a process during which a syncytium becomes a group of cells delimited by membranes. This process is associated to transcriptional activation [Anderson & Lengyel 1979] [Edgar & Schubiger 1986]. Interestingly during this process, nuclei get deformed by bundled microtubules and the nucleus stiffens during this process as well. If the stiffening is prevented the shape change cannot be maintained. Before this shape change, microtubules polymerization is responsible for nuclear envelope fluctuations [Hampoez *et al.* 2011]. From a chromatin point of view, nuclear envelope fluctuations are transmitted to the chromatin and induces its movement [Hampoez *et al.* 2011] like it

was described in adherent cells [Makhija *et al.* 2015]. Interestingly in this case, reducing LINC-MTs interaction enhanced the fluctuations showing that the LINC complex buffers the polymerization induced forces [Hampoelz *et al.* 2011]. In the end, when nuclei undergo transcriptional modifications they switch between a fluctuating phenotype to a more stable one fitting the general view that quiescent cells are characterized by a relatively more fluctuating/open chromatin state. Nevertheless, how these fluctuations impact gene expression and how it could be involved in differentiation is still unknown.

To sum up, microtubules are able to generate forces on the nucleus and impact its shape. Evidence show that it can also impact chromatin movements, especially by increasing their spatial fluctuations. At this stage, microtubules are good candidates for mechanical controlling gene expression and differentiation but this has not been completely shown yet.

3 Current Issues

Microtubules are able to generate forces in different ways and different contexts in adherent cells. Because studies have focused on the relationship between actin and nuclei only few examples have demonstrated that microtubules could potentially impact gene expression. Unfortunately, actin seems to be masking microtubules effect as it generates higher forces. In order to determine the contribution of microtubules one would need a single cell model where actin is less dominant and microtubules could be the ones leading the force generation on the nucleus. This kind of model could help to answer the questions associating microtubules and mechanotransduction.

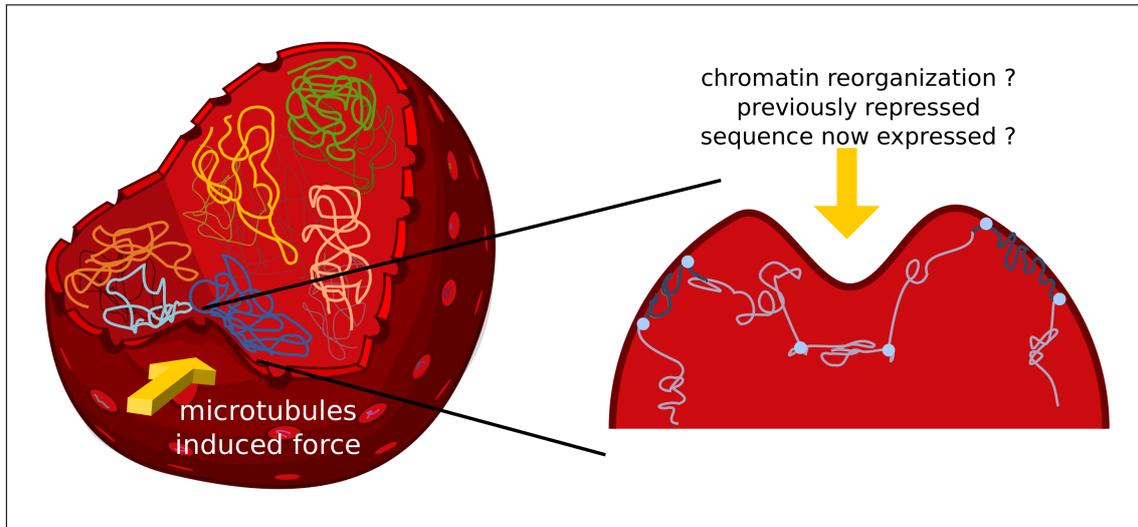


Figure 1.17 – How forces deforming nucleus can impact nuclear organization still remains unclear.

IV Adherent vs. non-adherent cells

Most of the data linking cytoskeleton and gene expression were obtained in adherent cells. Opposed to them, are the non-adherent cells, which only make very weak contacts with the substrate they are on. Lack of adhesion leaves the cell floating, like in the blood stream for instance. These cells are characterized by smaller size than adherent cells ($5\mu\text{m}$ vs. $50\mu\text{m}$) and a spherical shape, like adherent cells before spreading (figure 1.18).

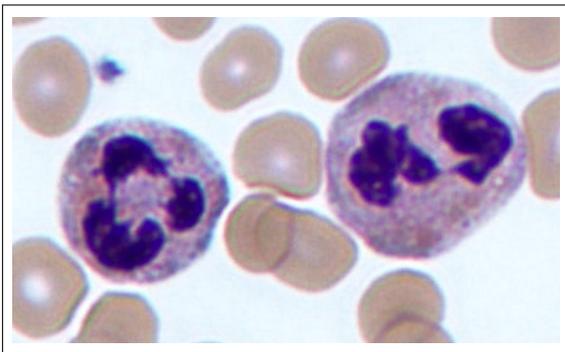


Figure 1.19 – Nuclear lobulations observed in neutrophils in a blood sample. Pale red cells are red blood cells.

One system of non-adherent cells changing identity is the hematopoietic stem cell. Looking at mature blood cells one can observe a great variety of morphologies: red blood cells are enucleated cells, platelets are torn out of a huge cell (the megakaryocyte), lymphocytes are rather small and some innate immune cells (myeloid cells) display a lobulated nucleus. Some of these nuclear morphologies changes involve microtubules

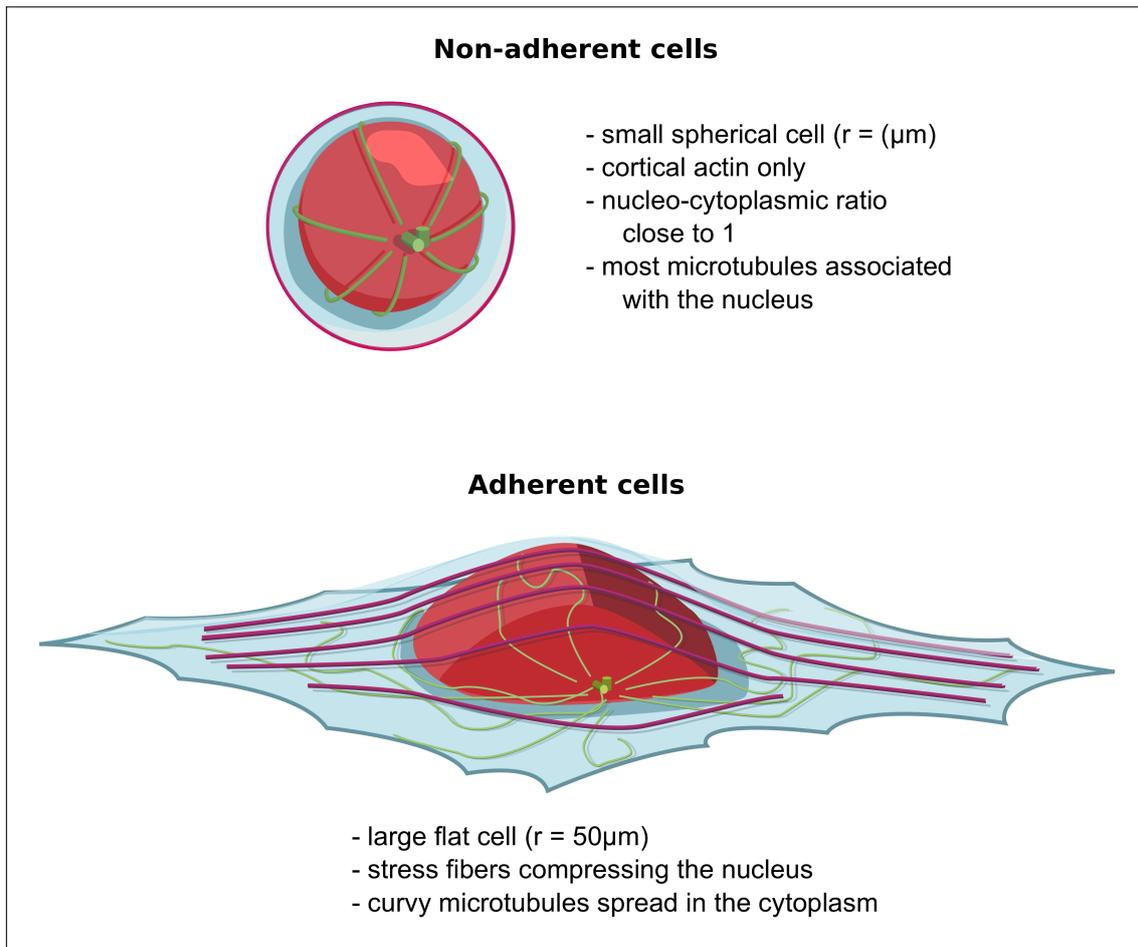


Figure 1.18 – Non-adherent and adherent cells display strong morphological differences. Their size differs by a factor 10, non-adherent cells only have cortical actin far from the nucleus and most of the microtubules interact with the nucleus whereas in adherent cells stress fibers are present and microtubules spread everywhere in the cell. These characteristics make the non-adherent cell a good model to study microtubules impact on nuclear shape.

as described previously [Bender *et al.* 2015]

[Kobayashi *et al.* 2016].

The case of the myeloid pathway, giving rise to granulocytes, is the most interesting in the context of chromatin organization because of their highly deformed nuclei (figure 1.19). Most of the data in this respect were obtained on neutrophils, the most abundant type of granulocytes in the innate immune system. Their characteristic lobulated nucleus was observed on blood samples and was historically thought to be multiple nuclei (figure 1.19). One potential involvement of these lobulations would be during migration. Indeed, those cells have to migrate from the blood stream to the tissue in order to prevent spreading

of pathogens. The consequences of the lobulations on chromatin organization have been poorly discussed but chromosome painting show that chromosome positioning is not random between different lobes [Bártová *et al.* 2001]. Adding to this, neutrophils are known to be the cells with the highest condensation level of chromatin [Sanchez & Wangh 1999] and could be linked to the nucleus shape. The mechanism involved during lobulation has not been well documented either. Nevertheless, it has been shown that lamins associated proteins like lamin-B receptor (LBR) and the LINC complex are needed for correct lobulation [Hoffmann *et al.* 2007] [Olins *et al.* 2009]. Supporting the role of microtubules, nuclear deformation is closely associated with centrosome position [Olins & Olins 2005] and perturbing microtubules during granulocytic differentiation from promyelocytic leukemic cells reduce their lobulation [Olins & Olins 2004].

Converging evidence suggests that microtubules dynamics changes between the stem state and mature cells and that it can influence chromatin positioning [Hampoelz *et al.* 2011]. Nevertheless, the link between microtubules, nuclear shape and chromatin organization has never been fully addressed. Neither has been determined if in systems where actin organization is more peripheral, microtubules could be involved in gene expression.

We’ve seen that the nucleus is a mechanoresponsive organelle, forces applied on it impact nuclear envelope properties that will in turn impact chromatin organization. These two aspects are intimately linked to chromatin expression state and can thus impact differentiation. In adherent cells the main actor able to generate forces on the nucleus is the actin cytoskeleton. By compressing the nucleus it can affect chromatin dynamics and if perturbed it can even trigger differentiation.

Because actin cytoskeleton generate high forces in adherent cells, microtubules contribution to nuclear mechanics and chromatin organization was never fully elucidated. Nevertheless, it is known that they are able to generate forces on the nucleus and participate to chromatin dynamics, during meiosis for instance.

To diminish the dominant effect of actin, non-adherent cells can be used as they display an actin organization less susceptible to impact nuclear mechanics. In this sense, I will show how hematopoietic stem cells constitute a well suited model to study impact of microtubules during differentiation, especially toward the myeloid lineage in which lobulated

cells can be found. It is nowadays well known how to select HSCs at specific differentiation stages and also how to differentiate them *in vitro* towards a chosen lineage. The best strategy is to split HSCs into populations representing the stem cells and early myeloid differentiated cells as it is the one giving rise to granulocytic cells. The populations selected have to be close enough in the differentiation process to be able to determine the intermediate state between the non deformed and deformed nucleus phenotypes. Once conditions were set up, I could correlate nuclear shape and chromatin organization and then determine that microtubules architecture could explain these changes. Ultimately, modulating microtubules organization during differentiation process impacted nuclear shape and chromatin organization. The present work show that microtubules can be established as a new actor for chromatin reorganization during myeloid differentiation.

Experimental Work

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I Results

1 Nuclear shape changes and chromatin reorganization occurs at an early myeloid differentiation stage.

Human umbilical cord blood hematopoietic stem and progenitors cells (HSPCs) are characterized by the expression of the surface marker CD34. Progenitors cells are distinguished by a high level of expression of the surface marker CD38. Thus it is possible to distinguish two populations: the CD34⁺/CD38⁻ which are the stem cells, and the CD34⁺/CD38⁺ the progenitors. The progenitors can be split in two populations, the one coming from the myeloid progenitor (MP), here selected by the expression of the surface marker CD33, and the one coming from the lymphoid B progenitor (LP) selected by the expression of the surface marker CD19 (figure 2.1A). We thus FACS sorted HSPCs in three populations in order to study their intracellular architecture: the CD34⁺/CD38⁻ cells representing the stem cells, the CD34⁺/CD38⁺/CD33⁺ cells representing the myeloid engaged cells and the CD34⁺/CD38⁺/CD19⁺ cells representing the lymphoid B engaged cells (figure 2.1B). We could observe that stem and lymphoid cells nuclei appear rather round compared to myeloid, which are bigger and display large invaginations in 2 dimensions (figure 2.1C). SUN-2 was used in order to visualize the nuclear envelope. In order to better describe these invaginations, nuclei images were acquired with a confocal microscope and 3-dimensional meshworks generated for analysis (See Material and Methods) (figure 2.1C). Nuclear volume was measured for each population and myeloid nuclei's show a significant higher volume compared to stem and lymphoid cells (figure 2.1D). The deformation of the nuclei could also be measured. Here again it is significantly increased for myeloid cells compared to stem and lymphoid cells (figure 2.1D). We then measured lamin A : lamin B intensity signal ratio, a parameter indicative of nuclear stiffness [Shin *et al.* 2013] (figure 2.1E). This ratio is increased between stem and progenitor cells (figure 2.1F) indicating that besides growing and deforming the nucleus becomes stiffer suggesting that forces applied to the nucleus have to be high or happen before stiffening.

To study the impact of these deformations on chromatin organization, we used a constitutive heterochromatin marker (H3K9me3) [Ugarte *et al.* 2015]. Both stem and lymphoid nuclei exhibit homogeneous peripheral signal distribution (figure 2.2A). On the contrary,

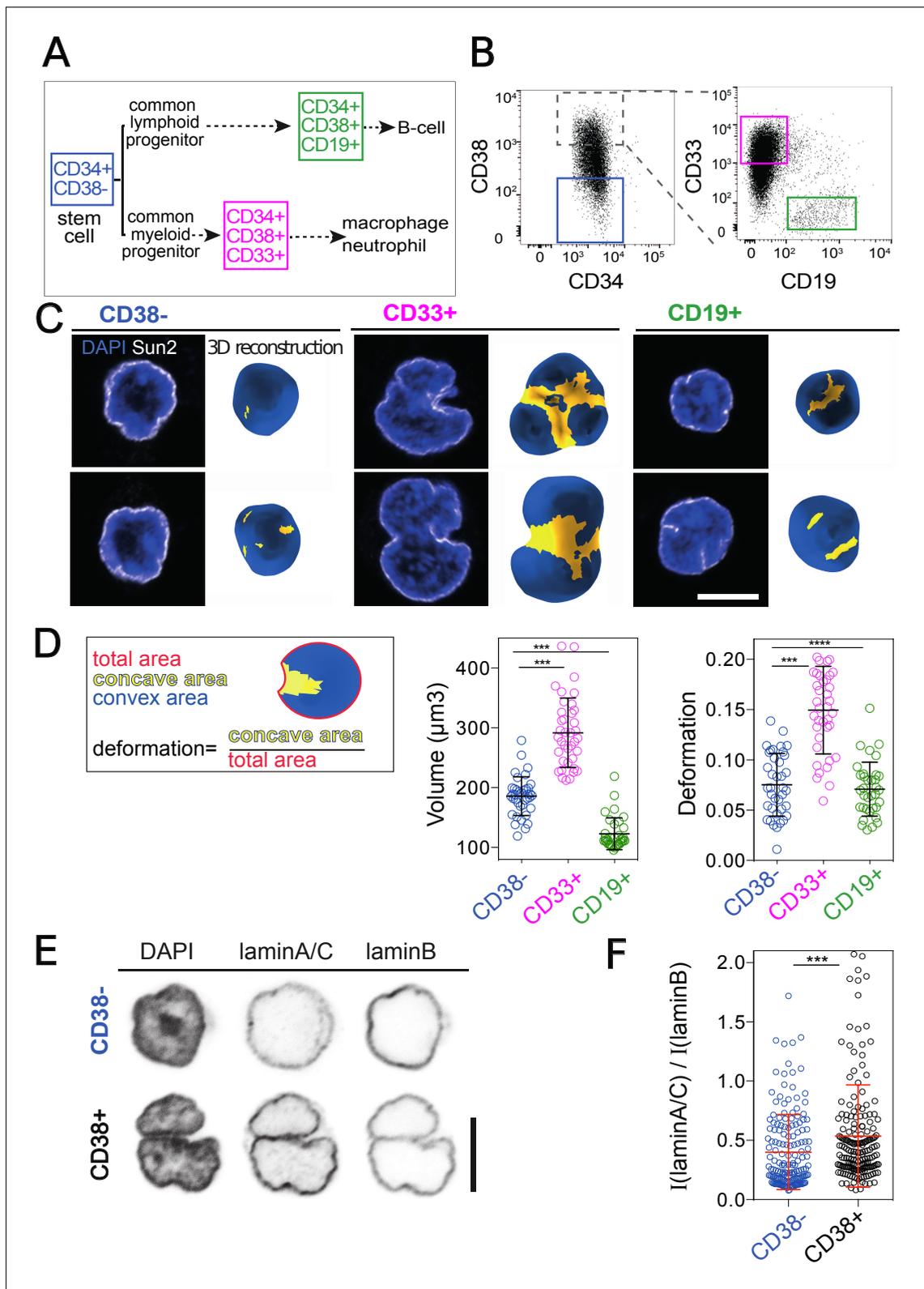
myeloid nuclei have more heterogeneous peripheral signal distribution (figure 2.2A). It was confirmed measuring the signal dispersion along the nucleus border (figure 2.2B, C) by computing the quartile coefficient of dispersion (figure 2.2D).

We also decided to analyze H3K27me3 position, another heterochromatin marker used to identify repressed genes during hematopoiesis [Ugarte *et al.* 2015] and that has been shown to vary during myeloid lineage commitment. We could see similar intensity heterogeneity as H3K9me3 which supports the idea that nuclear deformation can impact overall heterochromatin positioning. Unfortunately the weak signal intensity and difficulties to reproduce the observations did not allow further quantification (figure 2.3).

2 Cytoskeleton organization correlates with nuclear shapes.

We hypothesized that one of the members of the cytoskeleton family could be able to generate forces responsible for the deformations described. Striking changes in microtubules (MTs) architecture but not in actin oriented the analysis. Stem cells display a low number of MTs and a nucleus occupying almost the total volume of the cell, whereas myeloid cells have a higher number of MTs which spatial position correlates with nuclear lobulations (figure 2.4A). Myeloid cells also have much higher nucleo-cytoplasmic volume ratio compared to the two other populations (data not shown). In myeloid cells, MTs bundles running along the nuclear envelope could be observed (figure 2.4A, insets) and seem likely to interact specifically with the nucleus. All of the MTs seem to be nucleated from the centrosome. Interestingly this structure is internalized in the biggest invagination (figure

Figure 2.1 (facing page) – (A) Stem cells and progenitors used in this study are isolated using the CD34 surface marker. Upon differentiation, progenitors start to express CD38 in contrast to stem cells (blue). Progenitors engaged in the myeloid (magenta) and lymphoid (green) pathways express respectively the specific markers CD33 and CD19. (B) FACS gating strategy to isolate stem cells (further referred as CD38⁻, blue box), cells engaged in the myeloid (referred as CD33⁺, magenta box) and the lymphoid (referred as CD19⁺, green box) differentiation pathways. (C) For each population, two representative nuclei are shown in the left panel using the equatorial plane. Chromatin appears in blue (DAPI) and nuclear envelope in white (Sun2). Scale bar: 5 μ m. For each nucleus, the corresponding 3D reconstruction is presented in the right panel. Convex and non convex surfaces appear respectively in blue and yellow. (D) Nucleus deformation is the ratio of the non convex area over the total nucleus area. Nuclei are larger and more deformed in CD33⁺ cells (magenta, n=38) compared to CD38⁻ cells (blue, n=38) and CD19⁺ cells (green, n=35) cells. (***: p<0.001. ****: p<0.0001, Mann Whitney test). (E) Representative lamin A/C and lamin B immunostaining in stem cells (CD38⁻, blue) versus progenitors (CD38⁺, black). Inverted images of the equatorial plane are presented. Vertical scale bar: 5 μ m. (F) The lamin(A/C) / lamin B ratio is significantly higher in CD38⁺ cells (n=191) compared to CD38⁻ cells (n=169); ***: p<0.001, Mann Whitney test), indicative of a nuclear envelope stiffening upon differentiation.



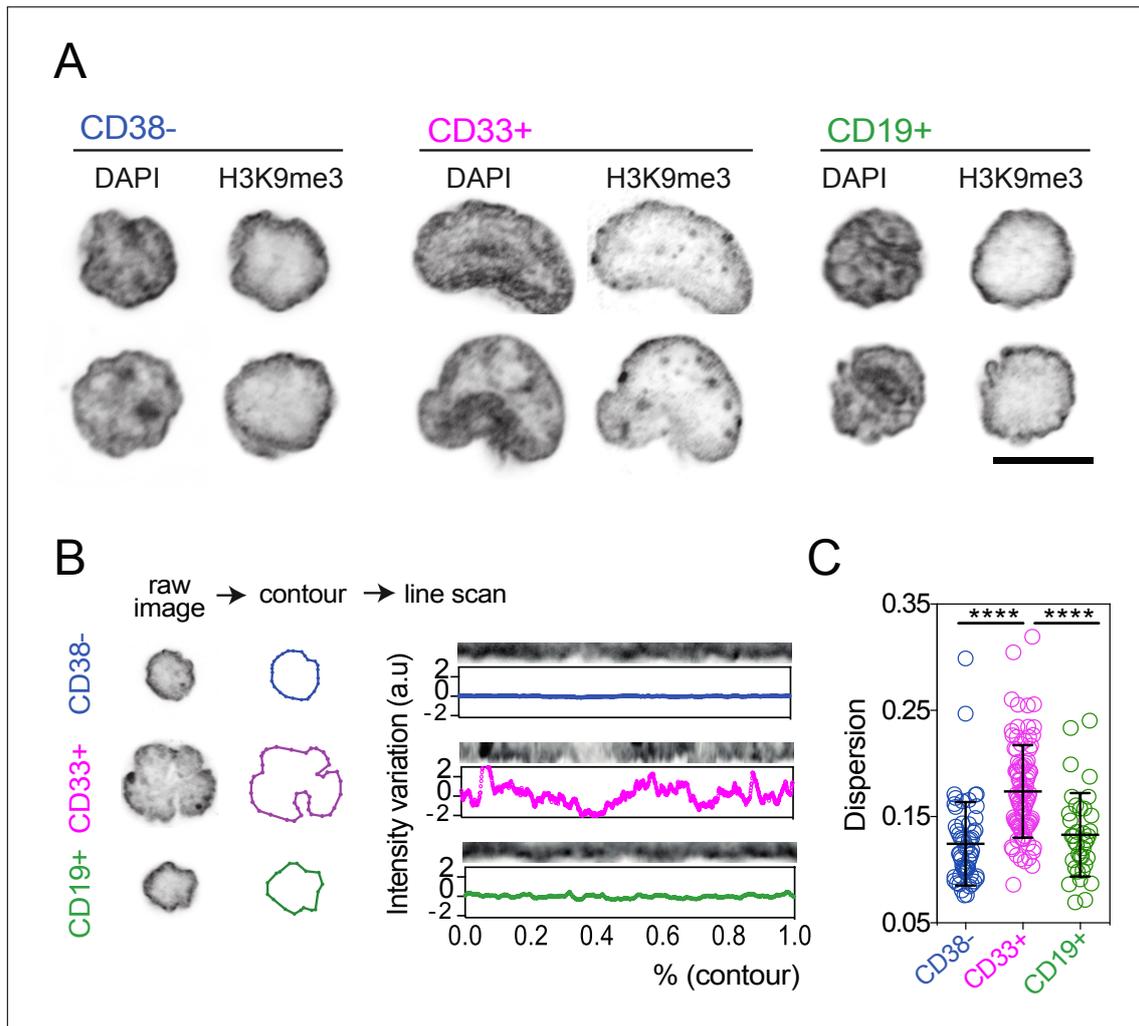


Figure 2.2 – (A) Spatial distribution of H3K9me3 in CD38-, CD33+ and CD19+ cells. For each population, two representative nuclei are presented. Inverted images of the equatorial plane of DAPI (left panel) and H3K9me3 (right panel) are shown. Scale bar: $5\mu\text{m}$. (B) For each cell, nucleus contour of the equatorial plane is extracted using the raw DAPI image (middle row). H3K9me3 intensity variations following nucleus contour are visualized and quantified (right row). Variations are used to extract the Dispersion parameter defined by the quartile coefficient of dispersion ($Q_{\text{disp}} = (Q_3 - Q_1) / (Q_3 + Q_1)$, where Q_1 and Q_3 are respectively the first and the third quartile of the intensity distribution). The dispersion parameter is higher in CD33+ ($n=98$) cells compared to CD38- ($n=58$) and CD19+ ($n=37$) cells (****; $p < 0.0001$, Mann Whitney test) indicating that H3K9me3 is homogeneously distributed at the nucleus periphery in CD38- and CD19+ cells, but becomes heterogeneous in CD33+ cells.

2.4C) and strongly support the idea that there is a strong link between the MTs network and the nucleus.

The fact that the centrosome gets closer to the cell center also means it goes further away from the cell membrane and makes it potentially less sensitive to external cues. We decided to measure in stem cells and progenitor cells the position of the centrosome relative

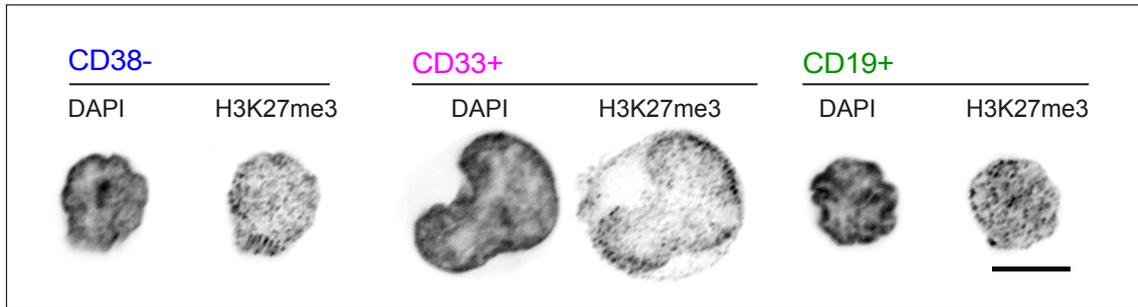


Figure 2.3 – For each population of CD38-, CD33+ and CD19+ cells, two representative nuclei are presented. Inverted images of the equatorial plane of DAPI (left panel) and H3K27me3 (right panel) are shown. Scale bar: $5\mu\text{m}$.

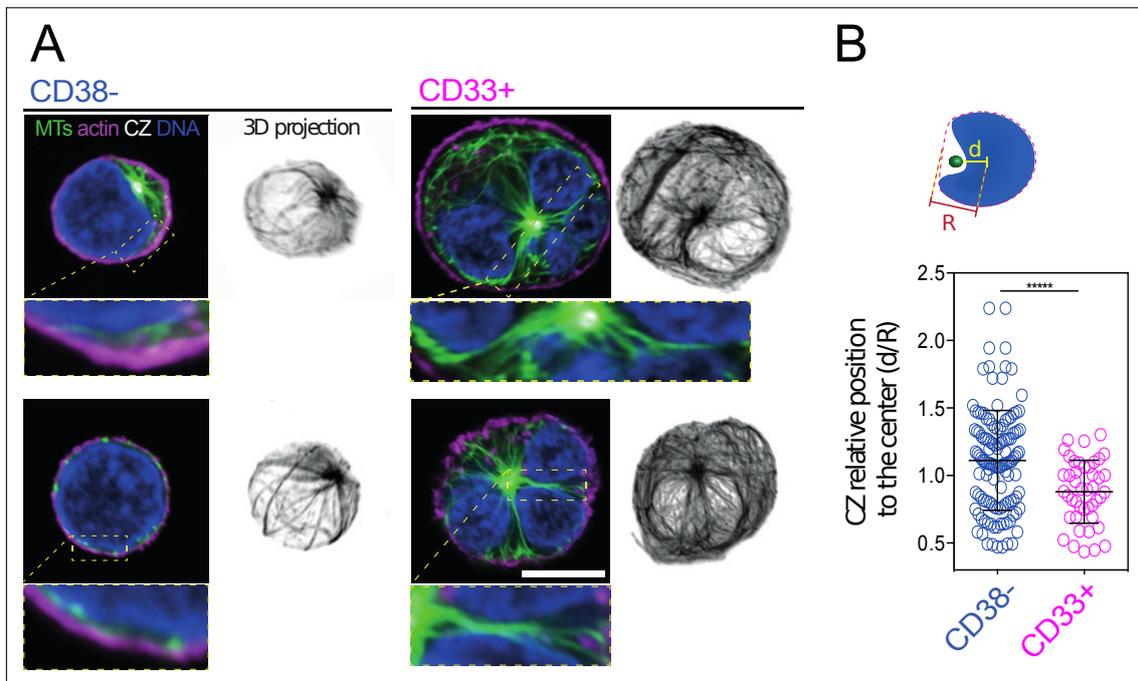


Figure 2.4 – (A) Two representative CD38- and CD33+ cells are shown in the left panel using selected Z stack with magnified insets. Microtubules appear in green, actin in magenta, centrosome in white and chromatin in blue. The corresponding 3D projection of microtubules is presented in the right panel. Scale bar $5\mu\text{m}$. (B) As schematized, centrosome-to-nucleus center distance (d) on nucleus center-to-nucleus convex envelope (R) ratio is calculated to extract the centrosome relative position to the center. This parameter is significantly lower in myeloid progenitors ($n=127$) compared to stem cells ($n=127$; ****; $p < 0.0001$, Mann Whitney test) indicating that the centrosome gets internalized upon differentiation.

to the center of the nucleus and the substrate, coated with different proteins. We measured the angle formed by the centrosome and the nucleus and we could show that stem cells, which have their centrosome located at the periphery of the cell tend to orient it towards the substrate whereas progenitors have their centrosome more randomly distributed (figure

2.5). Control condition with Poly-L-Lysine (PLL), which allows the cells to be fixed just after sedimentation, shows that the centrosome orientation in stem cells is not due to geometrical or physical factors (figure 2.5). This difference of centrosome orientation relative to the substrate suggests that nuclear deformation induced by microtubules could impact chromatin organization but also centrosome related phenomena like spindle orientation during mitosis.

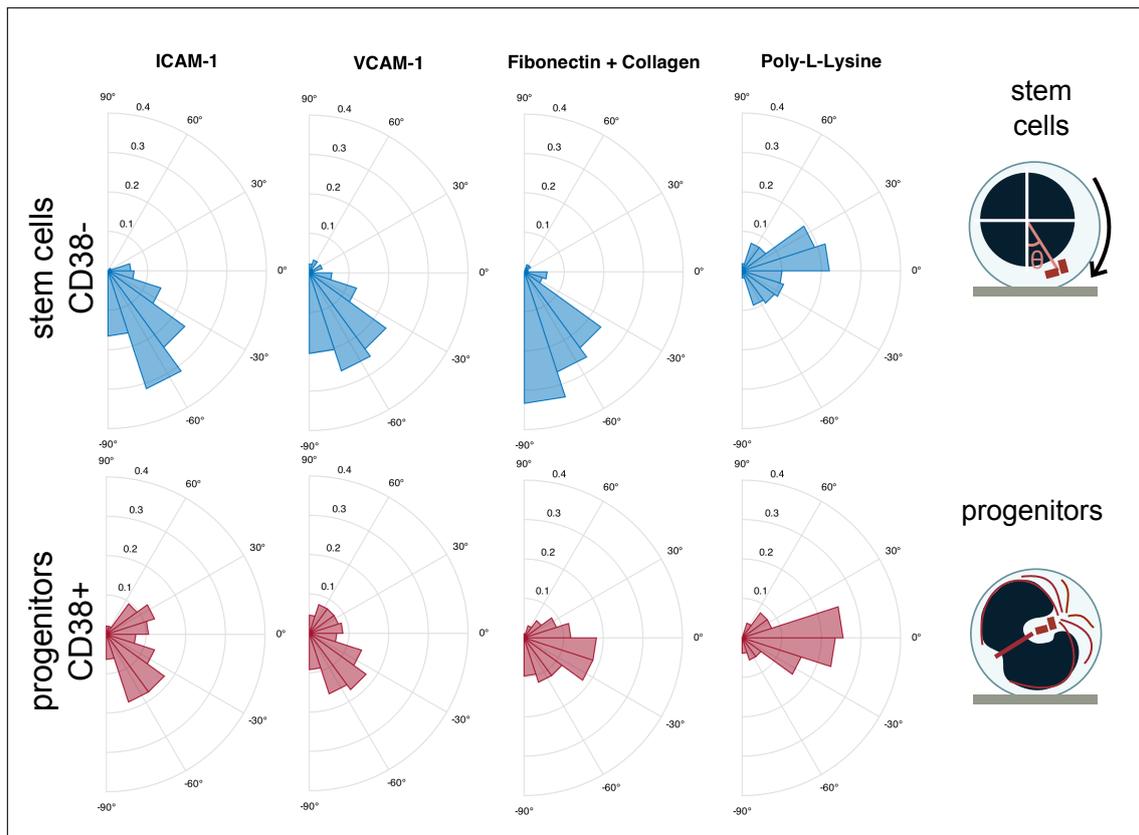


Figure 2.5 – Cells were seeded for 24h on coverslips coated with proteins known to favor lymphocyte adhesion (ICAM-1, VCAM-1, Fibronectin+Collagen) except for Poly-L-Lysine (PLL). Cells on PLL were seeded for 10 minutes and were used as a control to check if the mere difference in organization would lead to a biased sedimentation. Theta is the angle formed by the centrosome and the nucleus centroid in the yz plane. Stem cells (CD38-) have a centrosome oriented towards the substrate whereas progenitors (CD38+) have their centrosome more randomly positioned. Seeding on PLL coverslips does not recapitulate polarization suggesting an active process after sedimentation.

3 Nuclear deformation and chromatin reorganization are reproduced in culture.

To confirm that the correlation between MTs and nuclear shape is linked to stem cell differentiation towards the myeloid lineage, we allowed the freshly sorted stem cells to differentiate for 3 days in culture medium supplemented with growth factors (IL-3/SCF/G-CSF) and we analyzed their morphology each day (figure 2.6A). Quantitative analysis of the nuclear shape shows that volume and deformation level could reach comparable level than those of freshly sorted myeloid cells (figure 2.6B,C). Most interestingly cells at day 2 display a high heterogeneity of morphologies suggesting that the time window during which the architectural reorganization occurs is between day 2 and day 3. Supporting the hypothesis that nuclear deformation impacts directly H3K9me3 position, already deformed nuclei at d2 display heterogeneous intensity pattern at the nuclear periphery (figure 2.6D,E). At day 3, the dispersion becomes non distinguishable from freshly sorted myeloid cells (figure 2.6D,E). Strikingly when H3K9me3 signal intensity is plotted against the curvature of nucleus, regions of weak intensities correlates with regions of negative curvatures (figure 2.6F) and strongly support the hypothesis that nuclear deformation are responsible for heterochromatin reorganization. Interestingly, nuclear deformation measured in 2 dimensions (Shape Index) positively and linearly correlates with H3K9me3 signal dispersion (figure 2.6G). It allows to infer chromatin distribution based on nuclei shape and suggests that the deformation dependant chromatin reorganization is a continuous process.

Altogether this data show that HSCs myeloid differentiation system is a well suited model to study impact of microtubules perturbation on nuclear deformation and therefore on chromatin organization.

4 Time-lapse imaging of nuclear deformation.

To target microtubules and affect as little a possible the stem cell physiology we determined precisely what happens during the time window previously established. To achieve this, cells were cultivated for one day in normal differentiation conditions then placed in 25 μ m diameter poly-ethylene-glycol-diacrylamide (PEG-DA) microwells to perform time-lapse imaging of the nuclei between day 2 and day 3 of culture (figure 2.6G). Nuclei were stained

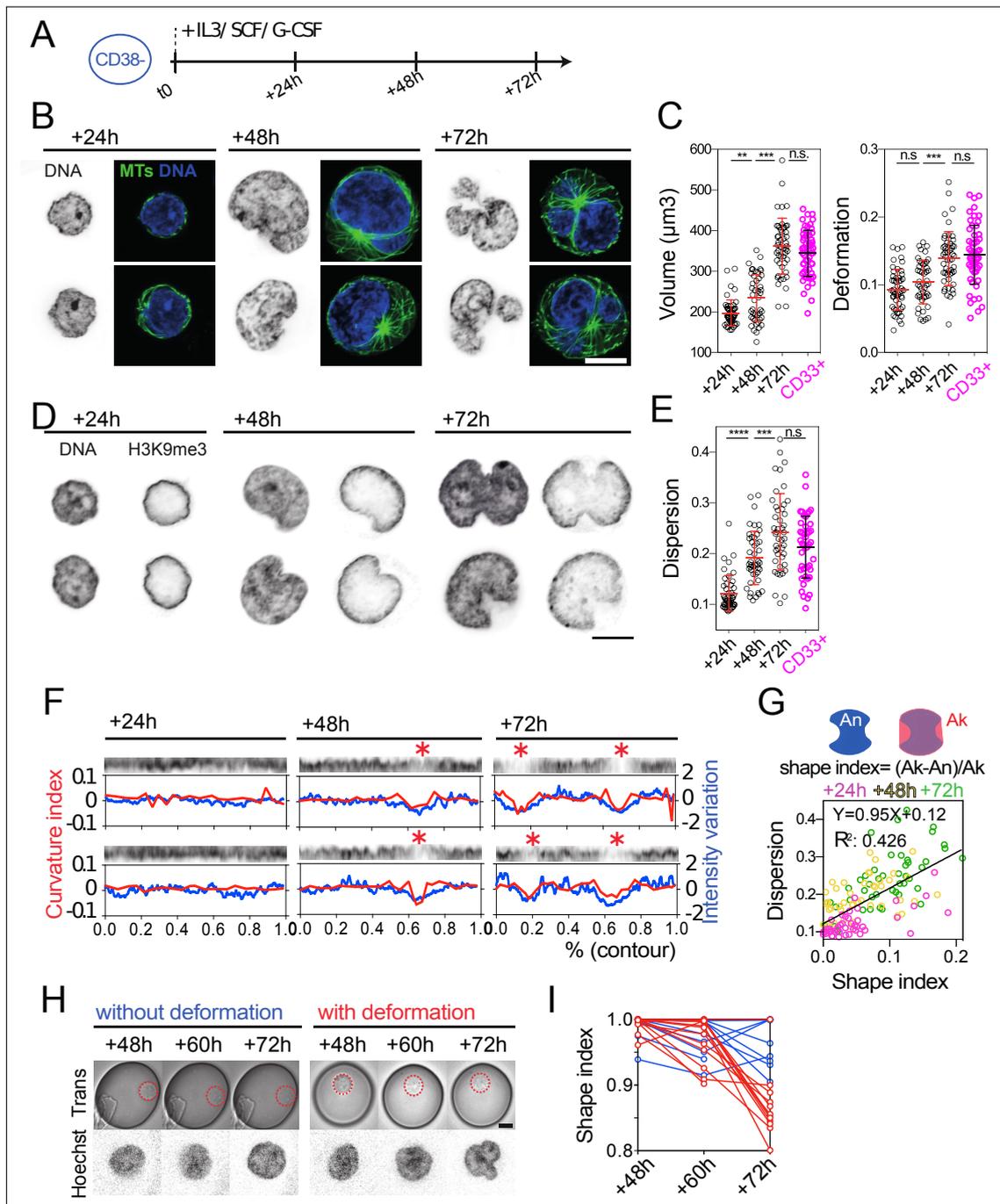
with DAPI 12 hours before imaging. Two types of behaviour could be observed: on the one hand a population of cells that keep a round nucleus, on the other hand a population of cells which nuclei go from a round to a highly deformed shape. Shape index of each nuclei was measured at each time point, a variation of 10% compared to initial value was considered as a deformation (figure 2.6H). This result shows that nuclear shape changes can be monitored in real time and that during this event the cell does not enter mitosis making it possible to perturb microtubules without affecting the division process.

5 Long-term time-lapse of migrating HSCs.

Unfortunately due to low temporal frequency and maximum 24h imaging period, we could not monitor nuclear shape before and after division. Nevertheless, imaging only with white light allows to obtain long-term movies of HSCs and study their behavior.

It is known that asymmetric division (regarding fate determinant) can occur in HSCs [Zimdahl *et al.* 2014] and that, as we have observed as well, their migratory phenotype changes during differentiation. We thus decided to track, upon division, daughter cells of HSPCs, to see if these asymmetric divisions could be associated to asymmetric behaviors in daughter cells. To increase the probability to observe asymmetric division we used a

Figure 2.6 (facing page) – (A) Isolated CD38⁻ cells are cultured and differentiated upon addition of IL-3, SCF and G-CSF cytokines. Cells are collected and analyzed at 24, 48 and 72 hours after cytokines addition. (B) The nucleus gets progressively deformed upon culture. For each time point, two representative cells are presented. Equatorial Z planes are presented. Left panel: Inverted image of chromatin (DAPI). Right panel: microtubules appear in green and chromatin in blue. Scale bar: 5 μm . (C) Nuclei volume and deformation increase to reach levels similar to freshly isolated CD33⁺ cells (24h, n=50; 48h, n=45; 72h, n=50; CD33⁺ n=55; n.s: non significant; **: p<0.01; ***: p<0.001, Mann Whitney test). (D) H3K9me3 redistributes upon culture. Two representative nuclei are presented for each time point. Inverted images of equatorial Z stacks of DAPI (left panel) and H3K9me3 (right panel) are shown. Scale bar: 5 μm . (E) Dispersion of H3K9me3 increases with time to reach at 72 hours a level similar to freshly isolated CD33⁺ (24h, n=54; 48h, n=51; 72h, n=33; CD33⁺ n=42; n.s: non significant; **: p<0.01; ***: p<0.001, Mann Whitney test). (F) H3K9me3 is lost from nucleus periphery where invaginations form. For each time point, two representative nuclei are presented. Upper row: H3K9me3 line scans following nucleus contour. Local intensity drops are highlighted with red asterisks. Lower rows: corresponding quantification of H3K9me3 intensity variation (blue line) and curvature (red line). (G) Shape index, as a 2D marker of deformation, is calculated with the nucleus area (A_n , blue) and convex envelope area (A_k , red) measured the on equatorial Z plane. Shape index correlates with H3K9me3 dispersion. Values measured at 24 (red) 48 (yellow) and 72 (green) hours of culture are plotted. (H) Nucleus deformation does not require cell division. Live-cell imaging of individual CD38⁻ cultured in 50 μm -large micro-wells. Cells are classified according to the final nucleus deformation: Blue: deformed, Red: undeformed. Upper row: transmitted light images were taken every 6 minutes and indicated time points are presented. Cells are underlined with magenta dashed lines. Scale bar 10 μm . Lower row: Corresponding and magnified Hoechst fluorescence images of the nucleus. Images were taken every 12 hours and indicated time points are presented.



population of HSPCs intermediately expressing the surface marker CD38. We could observe both symmetric and asymmetric behaviour mainly distinguished by the distance travelled by each daughter cell. Figure 2.7A illustrate an asymmetric division where one daughter cell starts to migrate whereas the other one stays non motile. Figure 2.7B illustrates a case where both daughter cells stay non motile, potentially keeping their stemness and finally figure 2.7C shows that a symmetric division but both daughter cells start to migrate.

This results suggests that indeed asymmetrical behavior can be found after division in the daughter cells, but couldn't be associated to asymmetrical distribution of fate determinant nor to nuclear deformation yet.

6 Microtubules perturbation impairs nuclear shape and prevent chromatin reorganization.

Taxol and nocodazole are the most used biochemical compounds to perturb MTs dynamics. Complete microtubules depolymerization induces cell contractility [Chang *et al.* 2008], leading to blebbing in this case, blebbs which contain part of the nucleus and thus impact its shape independently of microtubules. To resolve this issue, blebbistatin was used in conjunction with nocodazole to block the induced cell contraction. Adding nocodazole ($2\mu\text{M}$) and Blebbistatin ($50\mu\text{M}$) at day 2 of differentiation, led to significantly less deformed nuclei than those in the control condition (figure 2.8A). Use of blebbistatin alone does not prevent nuclear deformation excluding actin mechanical effect in this process. Similar results were obtained by adding a low concentration dose of Taxol (50nM) (figure 2.8A). Interestingly, in this condition microtubules seem unable to tightly interact with the nucleus. Since microtubules are attached to the LINC complex via dynein we decided to use Ciliobrevin ($100\mu\text{M}$) to affect their dynamics at the nuclear envelope level. We successfully managed to affect nuclear shape with the advantage of perturbing less other microtubules dependant processes (figure 2.8A). This result highlights the role of microtubules induced forces via dynein on nuclear shape during early steps of myeloid differentiation.

Most importantly, we wanted to see if affecting nuclear shape during differentiation could in turn affect chromatin organization. It appears that preventing nuclear deformation

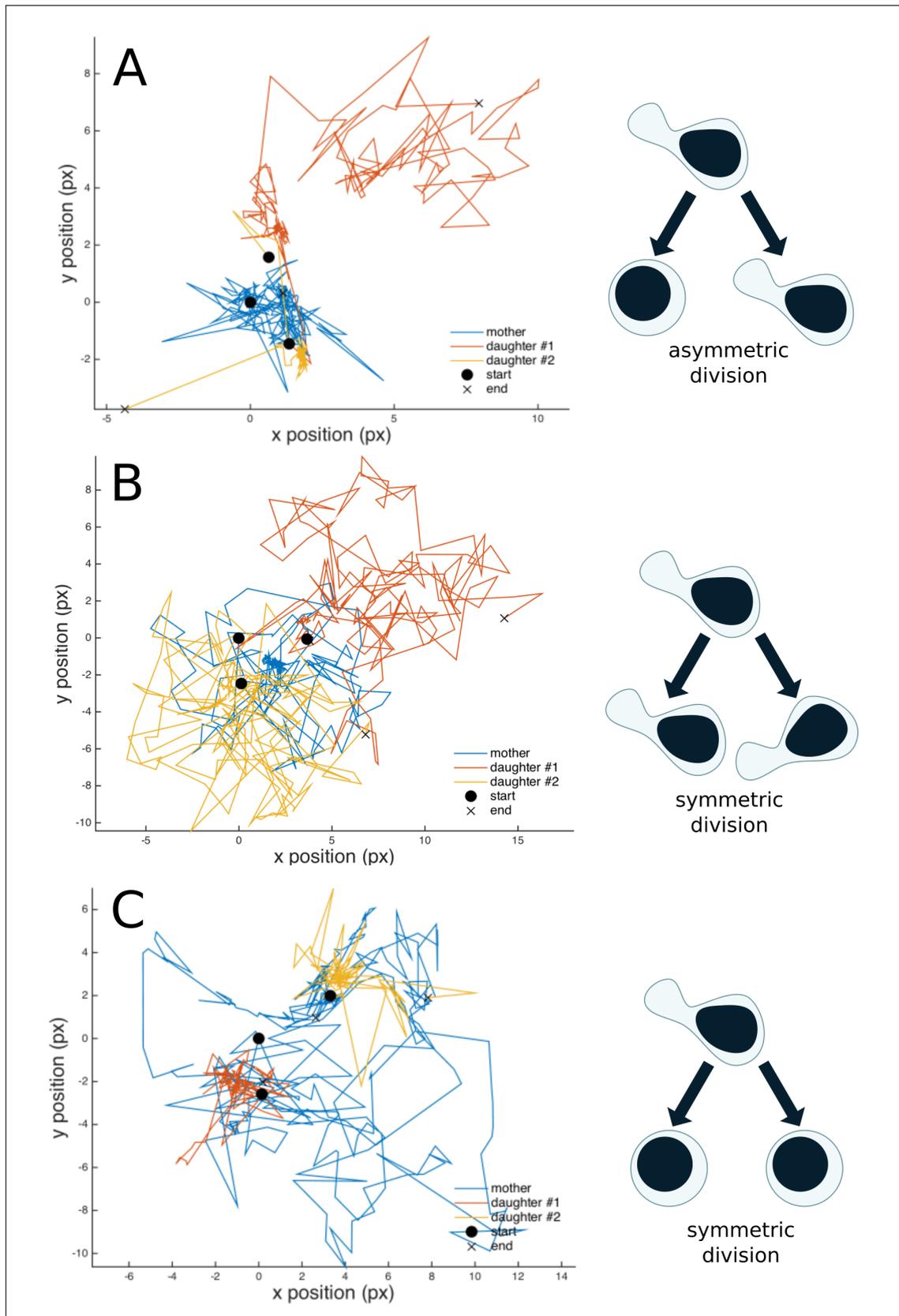


Figure 2.7 – HSPCs undergo transition between motile and non motile phases. When differentiating these transition become less frequent and the cell become non-motile. (A) shows an asymmetric division during which only one cell differentiate. (B) and (C) illustrate two symmetric division during which respectively daughter cells both keep their stemness or both differentiate.

prevents also nuclear peripheral signal intensity dispersion (figure 2.8C) in all described conditions.

When nuclear deformation is prevented, peripheral H3K9me3 distribution stays homogeneous. This results strongly support the idea that nuclear deformations are responsible for chromatin reorganization. In other words perturbing microtubules dynamics allows to keep a stem cell like H3K9me3 profile.

7 Microtubules deforming the nucleus are stabilized.

Interestingly, using low concentration of nocodazole (500nM) during differentiation did not prevent nuclear deformation. Most of the microtubules are depolymerized but the deformation happens anyway. Few microtubules remain and those are the ones running along the deformations (figure 2.9A). This observation suggests that the population of microtubules capable of nuclear deformation are somehow selected for their stability. Supporting this hypothesis, acetylated tubulin (a post-translational modification marking stable microtubules) could be found in the biggest invagination where the centrosome is located (figure 2.9B). This selection should happen before the deformation process occurs as nocodazole does not manage to prevent the deformation.

Altogether we show that nuclear deformation during early stages of myeloid differentiation is a microtubule-based mechanism. We show that MTs are capable of generating forces high enough to impact nuclear shape and that impairing their organization prevents nuclear deformation. Furthermore, we show that negative curvature at the nuclear envelope drives the transition of a homogeneous distribution of constitutive heterochromatin H3K9me3 marker at the nuclear periphery to a heterogeneous one. Altering microtubules organization with nocodazole or taxol treatment or dynein activity with ciliobrevin prevent nuclear deformation and subsequently H3K9me3 reorganization is abolished. Most probably, washing out the drugs at day 3 would lead to a delayed nuclear deformation at day 4 (figure 2.10).

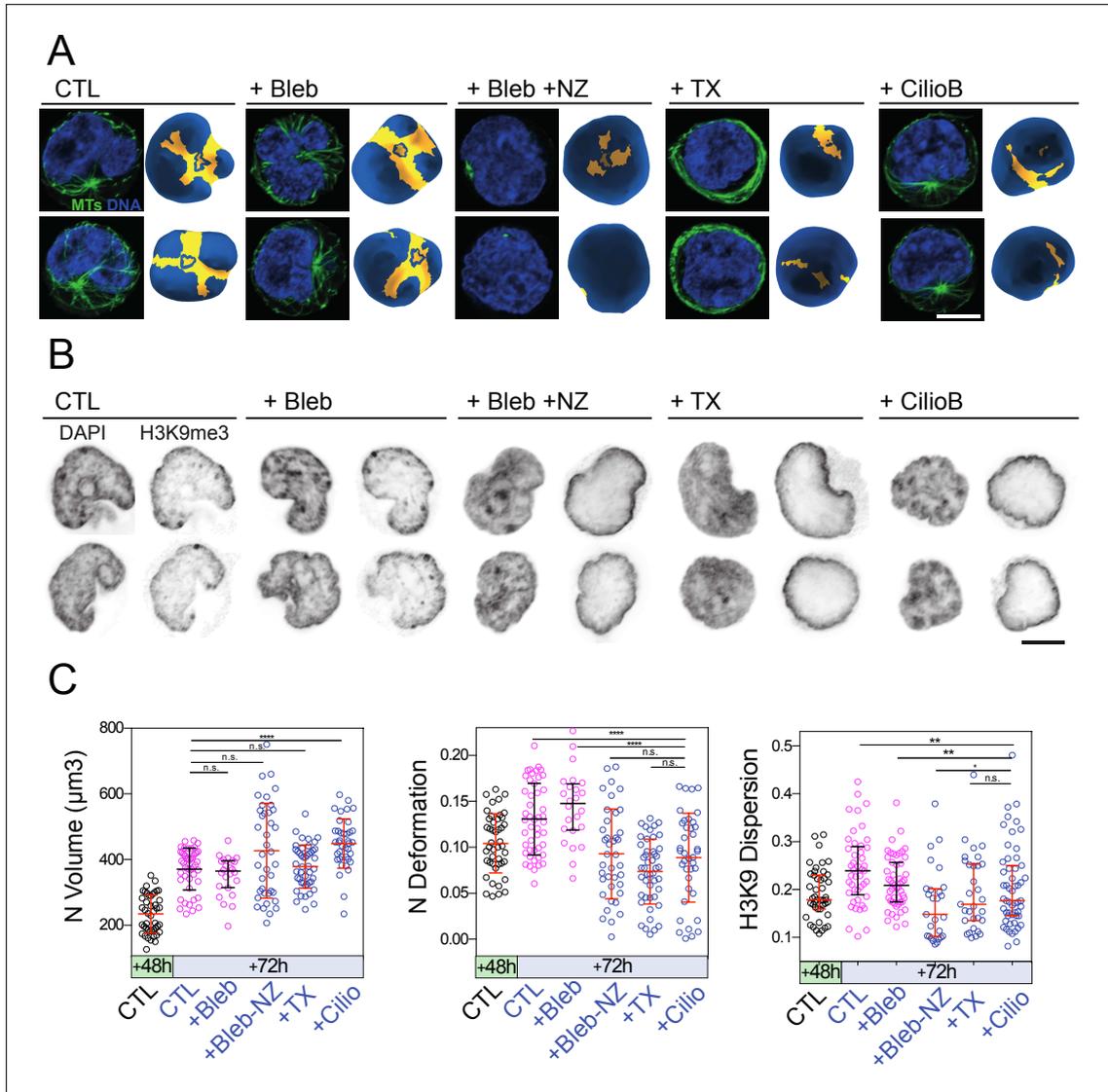


Figure 2.8 – Cells were fixed and analysed after 72 hours of culture. Microtubule drugs were added to culture medium between 48 and 72 hours. **(A)** Cytoskeletal organization and nucleus shape upon drug treatments. For each condition, two representative nuclei are presented. Left panel: equatorial Z plane; microtubules appear in green, chromatin in blue. Right panel: corresponding 3D reconstitution of the DAPI staining. Convex and non-convex surfaces appear in blue and yellow respectively. Scale bar $5\mu\text{m}$. **(B)** Microtubules perturbations during differentiation impair H3K9me3 redistribution. Inverted images of equatorial Z planes of DAPI (left panel) and H3K9me3 (right panel) are shown. Scale bar: $5\mu\text{m}$. **(C)** Quantifications of nuclear volume, deformation and H3k9me3 in the indicated conditions. Controls at 48h and 72h are presented.

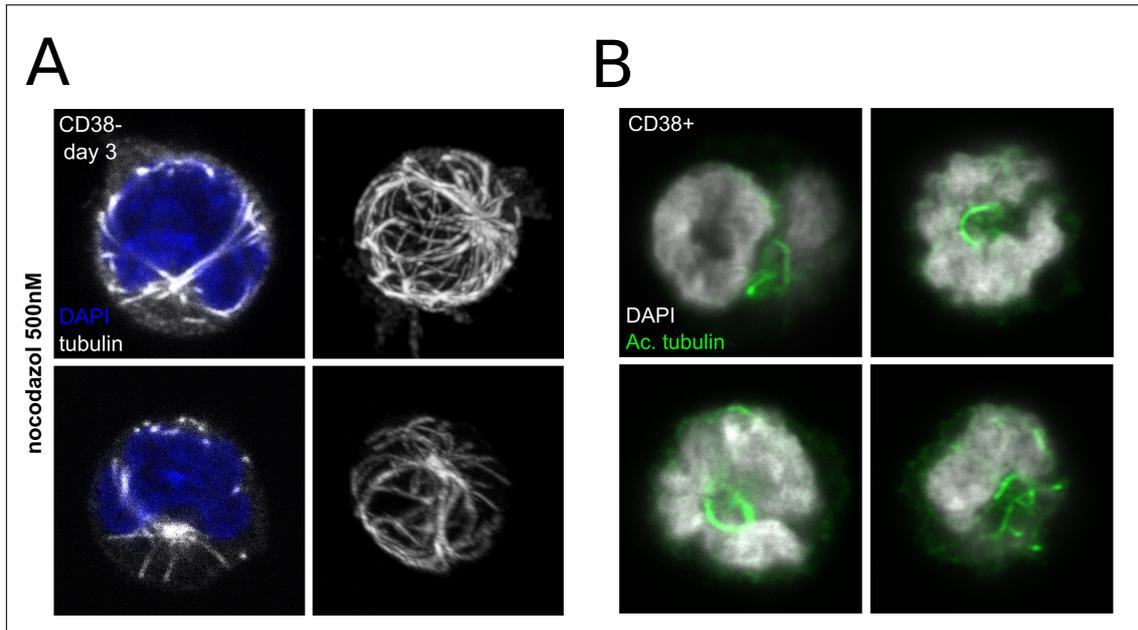


Figure 2.9 – (A) When differentiated in presence of a low concentration of nocodazole (500nM), HSCs' microtubules are still able to deform the nucleus although most of them have been removed. The remaining microtubules correlate with nuclear deformation and highlight a stable population of microtubules strongly linked to the nuclear envelope. (B) Acetylated tubulin, indicative of stable microtubules, can be found in the centrosome region and at the nuclear periphery.

8 Exploring dynein based mechanism: simulation

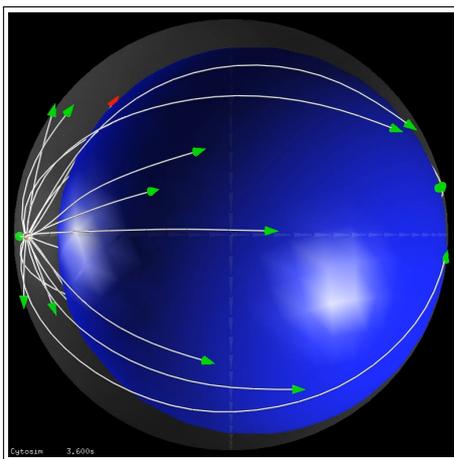


Figure 2.11 – Natively Cytosim can only generate non deformable objects.

Ciliobrevin effect on nuclear shape during differentiation shows that dynein is involved in nuclear shape but the mechanism by which it happens can't be deduced from the results obtained so far. To infer it, it is possible to use numerical simulation to explore different cell configuration and see if observations can be reproduced. Cytosim is a software developed by J.F. Nedelec made for cytoskeleton simulation. It can be used to simulate dynamic filaments, nucleators and molecular motors in both 2 and 3 dimensions. Unfortunately soft obstacles are not implemented. The only way to simulate a nucleus natively is to generate a non penetrable sphere (figure 2.11).

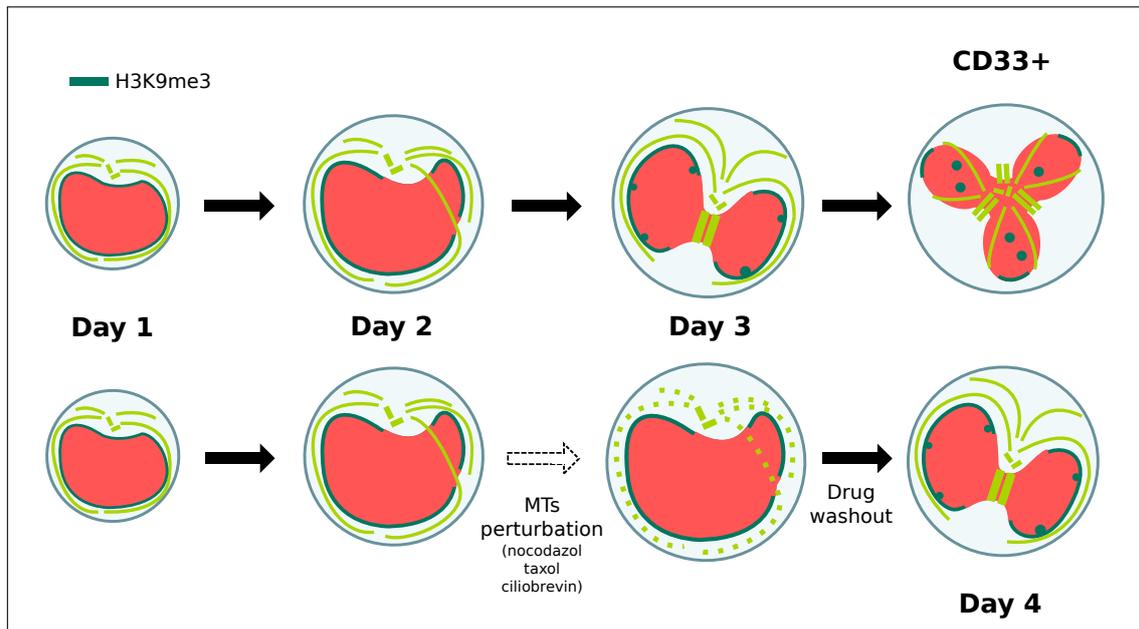


Figure 2.10 – During myeloid differentiation microtubules deform the nucleus causing heterochromatin marker H3K9me3 rearrangement. Perturbing microtubules dynamics during this process impairs nuclear deformation and prevents H3K9me3 reorganization.

A way to bypass this limitation is to generate several spheres. By doing so it is possible to reproduce a movable shape, however if the spheres are not maintained together the generated soft nuclei won't be cohesive meaning the spheres will move away from each other and microtubules will go through the structure. The size of the nucleus ($9\mu\text{m}$ diameter) represents here 70% of the cell volume ($10\mu\text{m}$ diameter).

In order to make the spheres hold together and prevent at the same time lateral penetration of microtubules in the shape, I used many filaments surrounding the structure and bound them together with cross linkers. This trick allows to generate a cohesive structure but parameters have to be correctly chosen so the mesh around the spheres is not too tight. If not, spheres can be ejected from the defined limit of the nucleus (figure 2.12).

Finally a centrosome can be added. It will nucleate dynamical microtubules that will undergo catastrophe (red arrow head) and rescue events (green arrow head) (figure 2.13). Parameters were chosen so that microtubules can go around the nucleus to the opposite side as observed in HSCs. In this case, the centrosome stays at the initial point. If dynein are added at the cell cortex, the centrosome move towards the nucleus and starts to form an invagination. Unfortunately the deformable-nucleus trick is limited as some microtubules

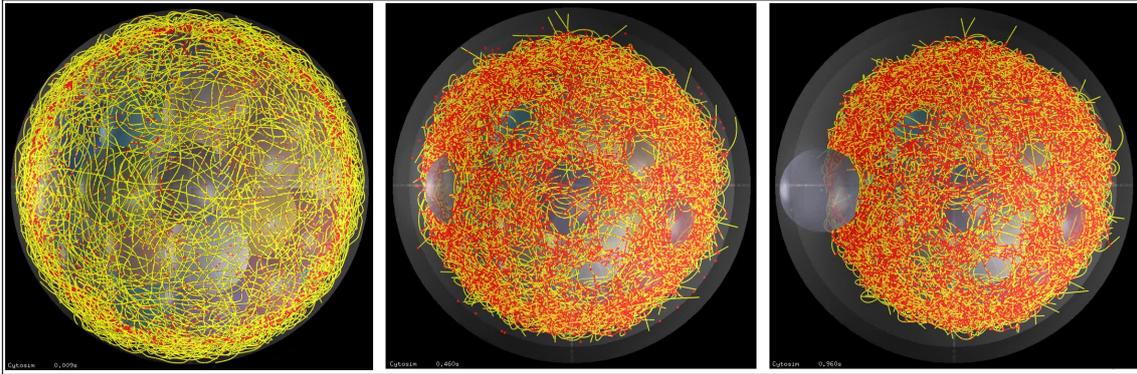


Figure 2.12 – By crosslinking filaments around a structure made of several spheres, it is possible to create a kind of deformable nucleus. The parameters have to be chosen carefully not to eject one or more spheres from the meshwork.

can penetrate the nucleus for unknown reason. Most probably because Cytosim was not designed to handle such structures and filaments interpenetration fails. For this reason longer simulation run are not relevant because microtubules starts to collapse in the nucleus as well as the centrosome. Another limitation of this approach is that its calculation speed is very low.

Nevertheless, these simulations show that dynein is needed to generate nuclear deformation. Here only the condition of dyneins at the cell cortex was tested and shows that even without LINC complexes it is possible to deform the nuclei. Further investigations would be needed to determine if dyneins at the nuclear envelope works as well (most probably) and what differences it makes compared to cortical ones.

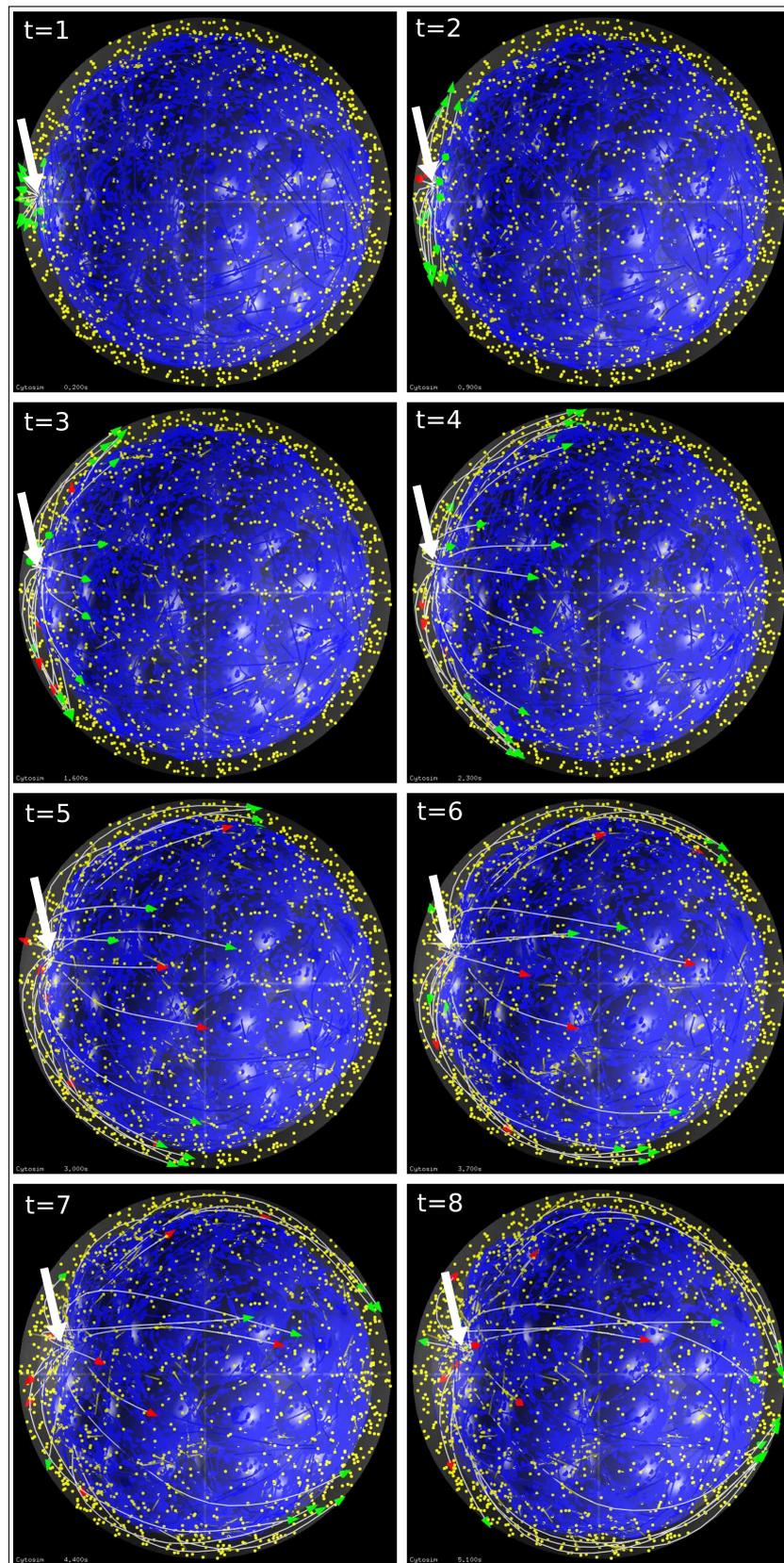


Figure 2.13 – Nucleus can be deformed by the action dynein located at the cell cortex. Arrow indicates the centrosome position. ‘t’ is an arbitrary time unit.

II Material and Methods

1 Sample preparation and cell culture

Human umbilical cord blood samples were obtained from the Saint Louis Hospital Cord Blood Bank (Paris, France) in accordance with French national law (bioethics law n° 2011-814) under declaration n° ?????? to the French Ministry of Research and Higher Studies. Mononuclear cells were separated with lymphocyte separation medium (Eurobio, Courtaboeuf, France), then CD34+ HSCs were isolated using MACS magnetic micro beads isolation kit and a QuadroMac Separator (Miltenyi Biotech, Paris, France) according to the manufacturer's instructions. Cells were either frozen in IMDM medium (Gibco) with 10% DMSO or used directly without freezing.

Cells were cultivated at 37°C in a 5% CO₂ humidified atmosphere. Cells were plated at a density of 40 000 cells/cm² in 96-wells plates in IMDM culture medium supplemented with antibiotics (Anti-anti, Sigma), 10% FBS and growth factors: human IL-3 1µg/mL (Peprotech), SCF 10µg/mL (Peprotech) and G-CSF 10µg/mL (Peprotech). One well was used per conditions.

2 Flow cytometry

Freshly isolated CD34+ or thawed cells were kept in 10mL IMDM medium supplement with antibiotics and 10%FBS for one night. Next day, cells were centrifuged at and resuspended in a 500uL solution of PBS/EDTA 2mM. For staining, 5uL per 10⁶ of antibodies (CD45-AF700 (BioLegend), CD38-PerCp5.5 (BioLegend), CD34-APC (BD Bioscience), CD33-PE (BD Bioscience), CD19-FITC (BD Bioscience)) were added for 30min at 4°C. Then cells were washed in 5mL of PBS/EDTA solution, and re suspended in PBS/EDTA at a final concentration of 4.10⁶ cells/mL. Sorting procedure was made on a FACS Aria II with DIVA software (BD Bioscience). After sorting, cells were centrifuged and re suspended in the desired volume of culture medium (with growth factors) to achieve correct cell culture density.

3 Immunofluorescence labelling

Poly-L-Lysine cover slips were prepared by putting the cover slips in the plasma machine for 30 seconds, then incubated with a commercial solution of Poly-L-Lysine (Sigma). Cells were allowed to sediment for 10 minutes at 37°C. Then, cells were fixed either with glutaraldehyde (Sigma) or paraformaldehyde (Sigma) depending on the structures stained. For cytoskeleton, a 0.5% glutaraldehyde with 0.1% Triton in Cytoskeleton Sucrose Buffer (10 mM MES pH 6.1, 138mM KCl, 3mM MgCl, 2mM EGTA, 10% sucrose) solution was used for 10 minutes. For nuclear staining, 3% paraformaldehyde in PBS solution for 20 minutes. Cells were washed with PBS, then permeabilized with 0.1% Triton for cytoskeleton staining, and 0.1% Triton 1% BSA for nuclear staining. Coverslips were neutralized with $NaBH_4$ for glutaraldehyde fixations, and NH_4Cl for paraformaldehyde ones for 15 minutes. Finally, 3% BSA/0.1% Tween in PBS was used as a blocking solution. This solution was used for all antibodies dilution. Cells were then stained with primary antibodies for 1h at room temperature (Rat Anti-YL1/2 (ABD serotech) 1 : 500, Rabbit Anti-Pericentrin (abcam) 1 : 2000) or at 4°C overnight (Rabbit Anti-H3K9me3 (abcam) 1 : 500). Coverslips were incubated with secondary antibodies or phalloidin (1 : 100 (Sigma)) for 1h at room temperature. Finally, DAPI 1 : 1000 (5ng/mL stock solution) (Sigma) staining was made for 5 min at room temperature in PBS. Coverslips were mounted with a Mowiol solution (Sigma).

4 Confocal Microscopy and 3D measurements

Images were acquired using a LSM 780 confocal microscope and ZEN software (Zeiss). Objective used was a 63x oil immersive (model), a 8x digital zoom was added. Each wavelengths were acquired separately with a 350nm z-step size to achieve proper reconstruction resolution. 3D projections of microtubules network was obtained using SOAX software (<http://www.cse.lehigh.edu/~idealab/soax/>). For nuclei, Image J plugin *3D viewer* was used to generate and export an isosurface of the DAPI threshold signal. The surface was then analyzed using MATLAB. It is imported as a mesh and smoothed using *openAndSmoothen* script (source name, MATLAB file exchange). Surface's main curvatures were calculated using the *patchcurvature* script (source name, MATLAB file ex-

change) and corresponding areas then measured. For centrosomes, positions were detected manually in the three dimensions from pericentrin images, an isosurface was generated and added to the nuclear reconstruction for further calculations. Images were generated with MATLAB, all data are plotted with Prism and statistical analysis (Mann-Whitney test) made with Prism.

5 Chromatin image analysis

Images were acquired using a LSM 780 confocal microscope and ZEN software (Zeiss). Objective used was a 63x oil immersive (model), a 8x digital zoom was added. Single slice were manually selected, post-acquisition, to maximize deformation in a 2D plane. Contour of the nucleus was manually detected (or automatically detected) on Image J. Straight views of the line selection were obtained with ImageJ straighten function. Line scans values and polygon vertices were extracted from ImageJ and analyzed with MATLAB. All data are plotted with Prism and statistical analysis (Mann-Whitney test) made with Prism.

6 Micro wells fabrication

Micro structured wafers were obtained by spin coating SU-8 photosensitive resist (company) on silicone wafers. First, a 5um thick layer (SU-3005) was made on the wafer and fully exposed to UV light (UV KUB2) for complete polymerization. Followed by a 50um thick layer (SU-3050) exposed to UV light through a plastic mask stuck on a glass to obtain pillars of 25um diameter. The obtained wafer was silanized with a gaz phase trichloro(perfluorooctyl)silane (Sigma). A negative PDMS (1 : 10, *reticulant* : *DMS*) mold of the wafer was then made, silanized, and used to obtain a positive mold of the original wafer. The PDMS pillars obtained are then placed on a 50mm u-Dish (Ibidi) and the inter pillars space was filled with poly(ethyleneglycol)diacrylate (PEG-DA) solution (80% PEG-DA length: 250 (Sigma) and 20% PEG-DA length 575 (Sigma)) with 1% hydroxymethylpropiophenone as reticulant. The whole montage was exposed to UV light, full power, for 15min in order to polymerize. The chip obtained was sonicated and in-

cubated with a collagen-fibronectin mix ($1\mu\text{g}/\text{mL}$ - $12\mu\text{g}/\text{mL}$) in PBS for 30min at room temperature, washed, and kept overnight in culture medium at 37°C to detoxify.

7 Time-lapse microscopy of nuclear shape

One day prior to imaging (24h), cells were incubated with $200\text{ng}/\text{mL}$ Hoechst (Sigma ?) diluted in culture medium supplemented with growth factors. Cells were seeded on top of the micro wells for 15min, washed, and the u-Dish was filled with fresh culture medium supplemented with Hoechst at $200\text{ng}/\text{mL}$. Images were acquired with a Yokogawa spinning disk module (CSU-X1) mounted on a Nikon microscope (Eclipse Ti), and using a 100x oil objective (Nikon S Fluor). Transmitted light images were acquired every 10 minutes, and DAPI images every 12h to avoid cell death. Nucleus contour was manually made using ImageJ, and values obtained plotted with Prism.

8 Long-term imaging of migrating HSCs

Two cover slips were plasmatized and coated with a fibronectin/collagen mix ($1\mu\text{g}/\text{mL}$ and $12\mu\text{g}/\text{mL}$ respectively). One cover slip was placed on top of the other separated by two bands of Parafilm (Bemis North America), then put briefly at 40°C so the Parafilm melted and stuck the two coverslips together. A cell solution of HSPCs expressing intermediate level of CD38 ($1000\text{ cells}/\mu\text{L}$) was injected in the formed canal. The set up was placed in a ChamSlide (Live Cell Instrument) and images were acquired every 10min for 48h. Tracking of cell division was made using Trackmate (ImageJ plugin).

9 Cytosim parameters

The following parameters were used for the simulations:

- cell radius: $5.5\mu\text{m}$
- spheres radius: $4.5\mu\text{m}$
- number of spheres: 24
- linking fibers:

- length: $10\mu m$
- rigidity: $1pN.\mu m^{-2}$
- microtubules:
 - growing/shrinking speed: $50/20\mu m.s^{-1}$
 - catastrophe/rescue rate: 0.4/0.1
- number of microtubules: 30

Discussion

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I Comments

1 Results summary

The present work aimed to characterize cellular architecture changes during early hematopoietic stem cell differentiation. We used human umbilical cord blood CD34+ cells which are known to contain the stem (CD34+/CD38-) and progenitor (CD34+/CD38+) cells. To determine if structural changes were lineage specific, the progenitor population was split into myeloid lineage engaged cells (CD34+/CD38+/CD33+) and lymphoid lineage engaged cells (CD34+/CD38+/CD19+). We could therefore show that nuclear

deformations could be specifically seen in myeloid cells but not in stem nor in lymphoid cells.

The deformation correlates with different organizations of heterochromatin (H3K9me3 marker) illustrated by the transition from a homogeneous to a heterogeneous distribution at the nuclear periphery. This result suggested that nuclear envelope deformation was involved in chromatin organization. Cytoskeleton architecture analysis have shown a sharp colocalization of microtubules, as well as centrosome's position with nuclear lobulation, that also have been observed in granulocytes [Olins & Olins 2005] and led to the hypothesis that microtubules generate forces on the nucleus capable of deforming it.

We managed to reproduce these correlations in culture. First, the deformations appear after 72h in differentiation conditions. Then, looking at H3K9me3, we could see that the signal becomes heterogeneous and follows nuclear envelope curvature. Negative curvature regions have lower signal intensity compared to positive curvature regions. Interestingly we could show that the amplitude of the deformation positively correlates with the dispersion of the heterochromatin signal. Variation of lamin B concomitant with local deformation have also been reported in other cell types [Gerlitz *et al.* 2013] supporting the hypothesis that deformation can induce local reorganization at the envelope level.

The contribution of microtubules was confirmed by perturbing their dynamics during this 72h differentiation period. Indeed, affecting microtubules organization, prevents nucleus deformation and thus no change in H3K9me3 distribution could be observed. Microtubules based deformation could have also been observed in promyelocytic leukemic cells differentiation upon retinoic acid activation [Olins & Olins 2004]. The fact that ciliobrevin affects nuclear shape puts dynein in the center of this mechanism and potentially the LINC complex, as it is known that dynein anchors microtubules to this complex. Supporting this hypothesis, over expression of SUN2 in lymphocytes have been shown to induce strong lobulation of the nucleus [Donahue *et al.* 2016].

This set of data shows that microtubules are able to generate nuclear deformation that can impact chromatin organization involved in gene expression changes during differentiation (identified here by the marker H3K9me3). We show that if we prevent nuclear deformation, chromatin reorganization does not happen.

2 Going finer in chromatin organization description

To strengthen the hypothesis according to which nuclear deformation affect chromatin organization, more heterochromatin marker or other techniques than immunofluorescence could be used to decipher the impact of nuclear deformations on chromatin organization. For instance, first euchromatin-heterochromatin pictures were obtained with transmission electron microscopy (TEM) (figure 3.1). This technique allows to see without any staining region of condensed chromatin. Moreover technical advances now allow to reconstruct 3D model from TEM stacks (tomography). It would drastically improve resolution of the nuclear shape description potentially highlighting changes not seen with confocal microscopy. It would allow to see how heterochromatin is organized at curved regions of the nucleus.

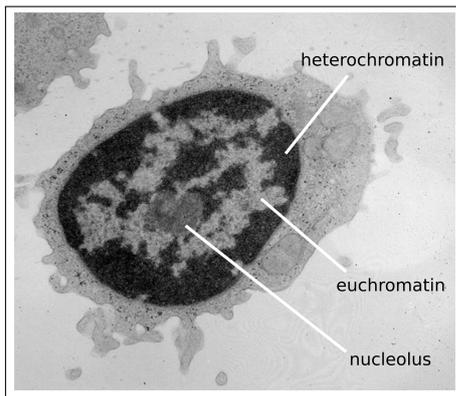


Figure 3.1 – Euchromatin and heterochromatin as they can be seen on electron transmission microscopy (ETM). [Korfali et al. - Mol Cell Proteomics - 2010; doi:10.1074/mcp.M110.002915]

Looking carefully at myeloid cells and *ex vivo* differentiated cells, H3K9me3 signal could also be observed as patches both at the periphery and inside the nucleoplasm. This phenotype, in which heterochromatin is distributed in the nucleoplasm and not at the periphery is observed in the rod cell of the retina, twchich have an ‘inverted’ nuclei’ [Eberhart *et al.* 2013] [Falk *et al.* 2018]. As it is thought to be due to the absence of lamins-chromatin interaction at the nuclear level, nuclear deformations as observed in the HSC case, could impact lamin ability to cluster chromatin. It is then freed from the periphery and could explain the internal relocalization. When the patches are observed at

the periphery the variability of the signal does not correlate any more with the curvature. It supposes that further processes occur and cannot be explained by current knowledge. Most probably nuclear deformation is a first trigger for further heterochromatin reorganization.

3 Another way to deform the nucleus

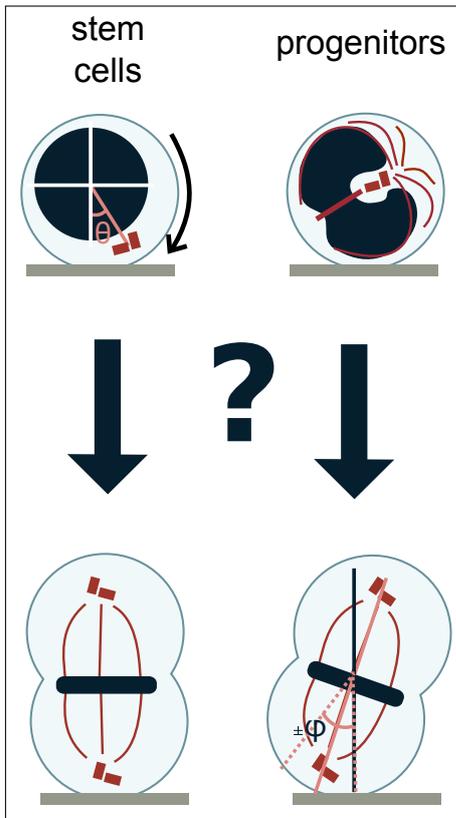


Figure 3.2 – Centrosome orientation towards cell’s substrate could impact spindle orientation during mitosis. In the stem cell case, spindle would be perpendicular to the substrate, whereas progenitor case leading to reproducible daughter cells positions after division. In the progenitor case, the orientation would be less precise and lead to differential spatial organization of the daughter cells.

We’ve shown that a sub-population of microtubules seems to be stabilized and responsible for nuclear deformation. This pre-stabilisation could lead to another mechanism of nuclear deformation. Indeed, we know that nuclear volume increases during differentiation. Thus, microtubules could be stabilized, then the nucleus grows, and those microtubules act as constriction point and the envelope deforms. Dynein and kinesin would only act as passive anchors in this case. To test this hypothesis one would need to increase nuclear volume at day 2. Two techniques can be used, either putting cells in a hypo-osmotic medium to provok water flux inside the nucleus [Kim *et al.* 2015] [Guo *et al.* 2017], or use trichostatin A (TSA) a histone deacetylase (HDAC) inhibitor. TSA provokes massive chromatin decondensation and is known to induce nuclear volume increase. The first approach can be cell destructive as it will change the concentration of all molecules in the cell.

4 Consequences of centrosome internalization

We could show that stem cells localize their centrosome mostly towards the substrate supporting the idea that centrosome interacts somehow with the membrane-substrate interface (figure 2.5). They can be two potential consequences of this prepositioning. First, clustering of fate determinants on the side of the centrosome could lead to two different daughter cells after mitosis [Zimdahl *et al.* 2014]. Secondly, the spindle alignment in regards to the substrate could be predetermined. If the cell is strongly polarized, the spindle can be

oriented perpendicularly to the substrate. After the division the two daughter cells will have different environments, one will stay in contact with the substrate the other will have one cell distance with the substrate, leading here again to an asymmetric division. In the case of progenitor cells, the spindle alignment with the substrate could be more random and responsible for a less reproducible asymmetric division (figure 3.2). Therefore, centrosome internalization via nuclear deformation could be a way to isolate the centrosome from external cues.

Moreover we observed as well that progenitors are less migrating on fibronectin/collagen cover slips (data not shown) compared to stem cells. Supporting this observation, it was shown that the motility of HSPCs is associated with its differentiation stage, the more they migrate the more they are differentiated [Moussy *et al.* 2017]. This behaviour switch could be potentially linked to the positioning of the centrosome with the nucleus. Indeed, it has been shown that centrosome is largely mobile in non-adherent motile cells [Crespo *et al.* 2014], trapping it close to the nucleus could then prevent migration.

5 Linking chromatin organization to differentiation

To obtain a better view of the differentiation process, transcriptomic will be used to determine if nuclear shape changes, when abolished, affect gene expression profile. Ideally, treated cells would have gene expression profile closer to stem cells than progenitors. To go further nuclear shape could also be compared with single cell transcriptome profiles either during differentiation or by externally inducing deformation.

Many techniques can be used to induce nuclear deformation like AFM or pipette micro aspiration, although both are low throughput. Development of microfabrication techniques now allow to work with high number of cells at once. Cells could be put in micro channels with irregular shape, doing so the cell would be deformed and could induce nuclear deformation. It would allow to study precisely the impact of externally induced nuclear shape deformation on chromatin. Nevertheless, comes the question of time scales. Indeed, nuclear deformation we observe happens on a 24h period. Although the dynamic is unclear at this point, it happens most probably progressively. Inducing externally nuclear deformation should take this aspect into account. Oscillating deformation and long term compression might lead to very different phenotypes.

6 Molecular tools limitation in primary cells

Unfortunately, with primary human cord blood HSC it is very difficult to use exogenous expression tools. Permanent gene expression (like viral transduction) is not possible as these protocols need cultivating cells for few days before being able to use them, meanwhile they differentiate. Other non permanent methods like transfection were tested. We could manage following some electroporation protocols to transfect HSCs with an efficiency of around 40% but here again these kind of protocols require at least 24h delay before the cell can be used, making them already differentiated for further analysis.

This constraint made Hoechst the only way to monitor nuclear shape in real time. Both its cytotoxicity and the fact that blue/UV light, also cytotoxic, is used to illuminate it, did not allow high frequency acquisition. To keep the cell alive only 3 images could be acquired over the required 24h time period. Similarly the impossibility to express fluorescent tubulin makes it impossible to have a fine description of the deformation process.

Same problem appeared for microtubules perturbation. Putting two drugs at relatively high concentrations like we did with nocodazole and blebbistatin treatment during 24h seems quite harsh. Same goes for taxol although concentrations are relatively low compared to what is commonly used ($10\mu\text{M}$). In these conditions many cells have defective mitosis or are apoptotic. It means that for most of them physiological processes, even before differentiation are impaired. Similarly, with blebbistatin alone some cells are binucleated suggesting that the cytokinesis has failed. We show that the deformation happens within a cell cycle but what happens before is not clear. It takes 20h for the first cells to divide, so they should have divided at least once before drug addition but maybe not all of the cells had the time to do so. What happened to the remaining observed cells ? Are those cells so quiescent they did not divide thus even without microtubules the nucleus would not deform ? Or were they perfectly caught between two cell cycles ? Hard to say.

Nevertheless, in live imaging experiments we could not observe asymmetric division where a round nuclei gives one round and one deformed. The few observed were symmetric. Either two round nuclei emerged from a round nuclei or two deformed from one deformed and the deformation is always observed hours after the putative first division. These two aspects support the fact that nuclear shape changes are independent of mitosis.

Fortunately, ciliobrevin treatment have similar effect without blocking so drastically mitosis as no defective mitosis are observed under microscope (data not shown). Two hypothesis could explain that: either the studied event involves dyneins anchored at the LINC complex pulling on microtubules or microtubules could slide between them around the nucleus (figure 1.15). To answer this question it is necessary to modulate quantitatively LINC complexes expressed by the cell.

Another approach to impact nuclear shape would be to modulate nuclear mechanical properties by increasing lamin A/C expression level in cells. Lamins A/C promoter is known to be sensitive to retinoic acid (RA) [Okumura *et al.* 2000]. Exposing progeric cells to RA restore nuclear shape by increasing level of lamin A. RA treatment on stem cells between day 2 and day 3, as did for microtubules related drugs, could be a way to prevent nuclear deformation by stiffening the nucleus. Nevertheless, interpretation would be difficult on chromatin as lamin would potentially increase chromatin interaction with the envelope even without deformation.

Generally speaking using drugs on primary stem cells seems one of the biggest limiting point. Most of the drugs used to perturb specific events almost always have side effects and we usually chose to ignore them because there is no better solution. It might no be limiting on short time scale events but have important consequences if the molecule is active for a long time. For instance depolymerizing microtubules is known to trigger contraction [Chang *et al.* 2008] so it is not possible to isolated completely the effect of microtubules architecture as it will impact something else. Similarly microtubules stabilization with taxol could affect some signalling pathways. About RA, long term exposure would have potential strong consequences on the cell expression profile as RA is known to be involved in the development of the heart [Lee & Skromne 2014]. Long term treatment can have strong gene expression effect unrelated to normal differentiation. Mechanical treatment like the one exposed above about externally induced nuclear deformation raise the same type of questions. In the end, these side effects have to be taken into account if we consider events that occur at time scales approximating the cell cycle. This consideration makes it more difficult to establish reliable protocols for modulating differentiation as the exact time window during which the event of interest happens have to be identified as precisely as possible to avoid drug overexposure.

7 Umbilical cord blood specificity

Here we used cells from human umbilical cord blood. These cells are still foetal like cells which first mean that they have potentially different profile than adult stem cells. Cord blood cells are purely circulating cells, do not have the same environment as cells in the bone marrow. It would be necessary to check if the observations we made exist *in vivo*. Ideally it would require working on living mice and do intravital microscopy unfortunately these techniques would not achieve correct resolution to observe nuclear deformation. Most relevant model would be fixed mice tissue slices where antibodies and nuclei staining can be performed to identify hematopoietic stem cells and progenitors among the others cells. Other important specificity of human cord blood is that the stem cells found in it are *myeloid biased* meaning that although they are stem cells they preferentially orient their differentiation towards the myeloid lineage. It can be identified by the surface markers profile: CD34+/CD38- cells already express the surface marker CD33 [Knapp *et al.* 2018] (data not shown). That's probably why we could observe the deformation phenotype so easily in sorted cells and during differentiation. It particularly made impossible to follow CD33 expression changes during differentiation. That is why we could not make time-lapse imaging of the deformation combined with CD33 staining to correlate nuclear shape with a cell identity switch. Moreover, it is not possible with this model to see if perturbing microtubules dynamics can impact cell fate choice. Similarly to what have been done with mesenchymal stem cells, it would be possible to put HSCs in differentiation medium supplemented half for lymphoid differentiating factors and half for myeloid differentiating factors. Modulating microtubules organization, thus nuclear deformation, we could have thought to bias differentiation towards lymphoid cells.

II Discussion

1 Other consequences of nuclear deformation

Nuclear deformation could be necessary for other phenomenons than chromatin organization. Lobulation in granulocytes are thought be facilitate migration through pores. Indeed, in other species neutrophils (a subtype of granulocytes) display various lobulation types.

Some have hyper-lobulated others hypo-lobulated nuclei and even round nuclei, the later mostly in non vertebrate [Carvalho *et al.* 2015]. Ring shapes nuclei have also been observed in mice and in myeloproliferative disorder in human [Carvalho *et al.* 2015]. Nuclear segmentation could help to maintain nuclear envelope integrity during migration depending on the tissue cell density. If we consider a round ball squeezing it will alter the envelope as described previously but if it is already contracted only small blebs of organized chromatin remain and can pass through pores without deformation.

Lobulations can have consequences on diffusion inside the nucleoplasm. The lobes being linked by smaller volume, it reduces the probability of a particle moving from one lobe to the other. Chromosomes become isolated and information transduced to one lobe will not necessarily be communicated to the others. It was already shown that chromosome repartition in two lobed neutrophils is not random suggesting that chromosome partitioning has a role in cell function [Bártová *et al.* 2001].

2 Nuclear deformations are not restricted to myeloid lineage

Concerning fine nuclear shape changes, one question remains about CD19+ cells. Their nucleus although at first glance seems not deformed, it often displays ridges going from one side to the other and that are depleted of H3K9me3. Microtubules can still be found there, which suggests again a microtubule-based process. Frontally growing microtubules pushing the envelope until reaching the other side seems the most probable hypothesis compared to microtubules strangling the nucleus so much the lipid layer collapses to form a tube. It suggests that deformation induced by microtubules can have different origins than strangling. These structures remain deformations with high curvatures and could explain why they lack H3K9me3 signal. These kind of deformations have also been reported in adherent cells and are dependant of the cell differentiation state but the mechanism and the consequences remain unclear [Johnson *et al.* 2003].

CD19+ cells belong to the lymphocyte type but are already biased towards B type lymphocytes. During a collaboration (unpublished) with Nicolas Dulphy's lab from the Institut Universitaire d'Hematologie (IUH), we had the opportunity to study intracellular architecture of a subtype of T lymphocyte, CD8+ lymphocytes not expressing the NKG2C receptor, an activator of lymphocytes cytotoxic activity. Surprisingly we could

also observe high nuclear lobulation correlated with centrosome positioning (figure 3.3) and microtubules (data not shown) in those cells. These observations show that nuclear deformation can be found in the lymphoid lineage and suggests that the observations on chromatin organization made in this work are not restricted to the particular case of myeloid cells.

3 Nuclear deformation: a marker of gene expression

Generally speaking nuclear mechanics were demonstrated to impact differentiation. MSCs differentiation, for instance, was shown to be impacted by substrate stiffness: soft substrate bias differentiation towards adipogenesis, stiff substrate towards osteogenesis [McBeath *et al.* 2004]. Substrate stiffness is known to participate to cell contractility, thus to the forces applied to the nucleus. This result strongly supports the importance of nuclear mechanics in gene expression and as a driver of differentiation.

Already mentioned hPSCs colonies also support this hypothesis. Indeed cells go from a confined and weakly adhering phenotype to a strong adhering one, known to impact nuclear and chromatin organization, thus differentiation [McBeath *et al.* 2004]. Perturbing actin organization in these colonies can trigger loss of stemness markers highlighting the importance of nuclear mechanics in stem cells in the maintenance of their potency. [Närvä *et al.* 2017]

The second aspect highlighted by results on hPSCs, and supported by results on drosophila cellularization [Hampoelz *et al.* 2011], is the transition between a nuclear fluctuating state and a locked one as it was shown that compression of the nucleus reduce envelope fluctuations and chromatin dynamics. It suggests that the stem state needs these fluctuations to somehow maintain chromatin in a given state, reducing them would lead to a less dynamic chromatin organization reducing its potency [Lopes Novo & Rugg-Gunn 2016].

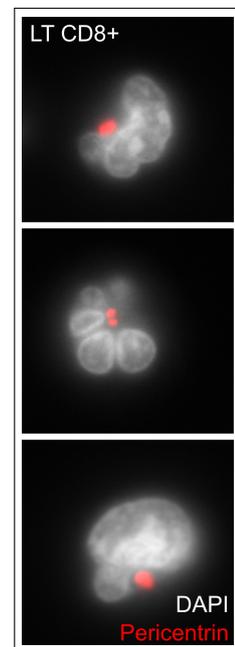


Figure 3.3 – LT CD8+ NKG2C(-) display highly invaginated nuclei and internalized centrosome. Due to their cytotoxic role it suggests that nuclear lobulation are linked with this function.

In this perspective, shaking the nucleoplasm could help to maintain chromatin in an open state keeping gene to several lineages available. It could also explain the large heterogeneity of expression profile observed in the stem cells as it could lead to increased chances of random binding of transcription factors to chromatin.

Interestingly, in the ciliates group, particular nuclear shape could be associated to specific developmental stage. These stages are also characterized by specific gene expression profile and strongly suggest that there could be a correlation between these profiles and nuclear shape [Wancura *et al.* 2018].

Nuclear deformation happens in physiological contexts but also in pathological ones. Diseases caused by lamin defects seem to strengthen the link between nuclear mechanics, chromatin organization and gene expression. Indeed, in progeric cells, the most studied laminopathy, cells exhibit abnormal nuclear shape [Uhler & Shivashankar 2018] that could be provoked by microtubules [Tariq *et al.* 2017], and is associated to particular gene expression profile characteristic of ageing [Scaffidi & Misteli 2008]. These results put nuclear mechanics and microtubules at the front line of a new gene regulation mechanism.

Second important pathological context involving nuclear shape is cancer. Indeed, it has been shown that cancerous cells exhibit abnormal nuclear shape and is even used as a criteria to determine cancer invasiveness [Chiotaki *et al.* 2014]. These abnormal shapes are frequently linked to defects in lamina constituents like lamins, emerin etc. These proteins are strongly involved in gene regulation and nuclear mechanics. On top of this, these nuclear deformations are also correlated with chromatin reorganization [Schirmer 2014]. It becomes clear that the nuclear envelope is not only a physical barrier between the DNA and the cytoplasm but is a critical component for gene regulation.

4 Microtubules: a new actor in mechanotransduction

Microtubules in hPSCs colonies cells have not been observed yet but their impact on nuclear mechanics probably follow the one of HSCs. Indeed, at first the cells are confined, a phenotype close to non-adherence. In this case, where actin is only located at the colony borders, microtubules could be able to generate deformations of the nuclear envelope. Then when the cell starts to spread, actin starts compressing the nucleus and microtubules effect is masked. The predominance of actin could explain why microtubules effect on gene

regulation have been poorly described yet or maybe the phenomenon in which microtubules are involved still remain unknown.

Fortunately, using non adherent stem cells like HSCs it is possible to lighten the contribution of actin. In this extreme case, microtubules are the only ones able to generate forces on the nucleus. That is most probably the reason why, even upon differentiation, microtubules are the one constraining the nucleus. The important fact is that, microtubules now appear as much competent as actin to regulate gene expression.

It has been observed that cancerous cells exhibit amoeboid like migration [Clark & Vignjevic 2015], similar to the one observed in non-adherent cells. If those cells adopt an non-adherent like behaviour it suggests that their microtubules could be interacting more with the nucleus, as opposed to what it would have been in a healthy, spread cell. The link between microtubules, nuclear envelope and chromatin organization could thus be of high interest in this pathological context and could help decipher how gene misregulation happens.

For instance, taxol is widely used as a chemotherapy drug to treat cancers. Its efficiency was thought to be due to its ability to prevent cell division of cancerous cells, by affecting mitotic spindle assembly. As these cells divide more often than healthy cells, it should reduce their number by inducing their apoptosis. This hypothesis comes from experiments on cultured cells and is indeed correct. Nevertheless, in tumours the mitotic index (the proportion of dividing cells) does not allow to validate this hypothesis: the number of dividing cells is too low compared to the efficiency of paclitaxel to confirm that the drug effect is due to mitosis defect [Weaver 2014]. So where does the taxol efficiency as a chemotherapy drug comes from ? Maybe the answer lies in its ability to reduce microtubules dependant force on the nucleus preventing undesired chromatin reorganization, thus gene misregulation.

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Rôle de l'interaction entre le noyau et les microtubules dans la régulation de l'organisation de la chromatine dans les cellules souches hématopoïétiques

Résumé : Les cellules souches hématopoïétiques sont caractérisées, comme chaque cellule souche, par leur capacité à s'auto-renouveler et à se différencier, ce qui leur permettent de participer à l'homéostasie du système sanguin. Comment ces cellules souches entrent dans une voie de différenciation ou une autre est encore mal compris mais un nombre croissant d'éléments obtenus dans différents systèmes soulignent l'importance des signaux mécaniques dans ce choix. Les signaux physiques du microenvironnement de la cellule peuvent-être transduits au noyau par l'intermédiaire du cytosquelette, qui va alors moduler l'organisation de la chromatine et donc l'expression des gènes : c'est la mécano transduction. Plusieurs études illustrent l'importance du cytosquelette d'actine dans les cellules adhérentes dans ce phénomène mais le rôle d'autres éléments du cytosquelette, comme les microtubules, reste encore mal connu. De plus, la mécano transduction dans les cellules non-adhérentes a peu été étudiées, cellules où l'organisation de l'actine ne permet pas de générer des forces sur le noyau. Le travail présenté ici montre que les microtubules peuvent déformer le noyau et réguler l'organisation de la chromatine dans un système de cellules souches non adhérentes, les cellules souches hématopoïétiques (CSH). **Mots-Clés :** mécanique noyau, microtubules, organisation chromatine, mécano-transduction, cytosquelette

Interplay between the nucleus and the microtubules: role in the regulation of chromatin organization in hematopoietic stem cells

Abstract: Hematopoietic stem cells are characterized, like every stem cells, by their self-renewal and differentiation ability so they can sustain mature blood cells populations. How stem cells engage in one or the other path is poorly understood but increasing number of evidence in different stem cell types highlight the importance of mechanical signal integration. Physical cues from the environment can be transduced to the nucleus via the cytoskeleton, to impact chromatin organization and therefore gene expression: this process is called mechanotransduction. Many studies bring light to the importance of the actin cytoskeleton in adherent cells in this process but very little is known about the contribution of microtubules in this process. Moreover, even less is known about mechanotransduction in non adherent cells, in which actin organization is likely to have smaller impact than in adherent cells. The present work show that microtubules can impact nuclear shape and chromatin organization in a system of non-adherent stem cells, the hematopoietic stem cells (HSCs). **Keywords:** nuclear mechanics, microtubules, chromatin organization, mechanotransduction, cytoskeleton
