ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent adult stem cells able to self-renew and differentiate into several cell types within mesenchymal origin, which can be collected from adult mesenchymal tissues, and also from extra-embryonic tissues. The latter constitute a good source of MSCs, being more *naïve* and having a more proliferative potential than MSCs from adult tissues, features that make umbilical cord matrix MSCs an appealing cell type for the generation of induced pluripotent stem cells (iPSCs).

The generation of human iPSCs, namely from human MSCs, has been reported, although with a low efficiency. It is known that pluripotent stem cells and their nuclei possess distinct elastic properties from differentiated and adult stem cells (and respective nuclei). We hypothesize that, by modulating the rigidity of MSCs, it may be possible to enhance the reprogramming efficiency using a lentiviral vector encoding pluripotency factors.

The nucleus is mechanically coupled to cytoskeletal elements by the LINC (Linker of Nucleoskeleton to Cytoskeleton) complex, thus mechanical forces from the extracellular matrix can be transmitted through the cytoskeleton to the nucleus. Depending on substrate stiffness, the nucleus is under more or less tension, eventually being possible to modulate its rigidity by culturing the cells on platforms with distinct degrees of stiffness.

Here we demonstrated that MSCs plated on substrates with distinct range of rigidity showed different degrees of efficiency to fully reprogram. Moreover, it was shown that maintaining MSCs on specific substrates enhanced the expression of pluripotency genes. The effect of substrate rigidity on the cells was evident when chromatin compaction and nuclear area were analyzed. Thus, nuclear euchromatic and heterochromatic content ratios and area could be modulated, suggesting that nuclei were subjected to mechanomodulation. Differences were also observed in what concerns the size of Focal adhesions (FAs) and the assembling of actin stress fibers.

Taken together, our results suggest that it is possible to improve the reprogramming process by modulating the substrate rigidity, and that the mechanism responsible for this improvement could be intimately related with the mechanomodulation of the nuclei.

The enhanced generation of human iPSCs cells using substrates with defined stiffness indicates that the current cell reprogramming protocols can be substantially improved by seeding the cells on such substrates. Thus, by improving the efficiency and kinetics of iPSCs generation, future strategies may be further explored using non-integrative reprograming delivery strategies, considered safer for putative future clinical applications.

Keywords: MSCs, mechanotransduction, iPSCs, reprogramming efficiency

RESUMO

As células estaminais mesenquimais (MSCs) são células estaminais adultas, multipotentes, capazes de se auto renovar e diferenciar em diferentes tipos celulares dentro das linhagens de origem mesenquimal. A colheita de células estaminais mesenquimais é feita a partir de tecidos mesenquimais e também de tecidos extra embrionários. Estes últimos constituem uma boa fonte de MSCs, sendo estas mais *naïve* e com maior potencial de proliferação do que MSCs de tecidos adultos, características que fazem com que MSCs da matriz do cordão umbilical sejam um tipo celular muito apelativo para experiências de reprogramação.

A geração de células estaminais pluripotentes induzidas (iPSCs), nomeadamente a partir de MSCs, tem sido documentada na literatura por diferentes autores, no entanto sempre associada a uma baixa eficiência. É sabido que células estaminais pluripotentes e os seus núcleos têm propriedades elásticas distintas daquelas apresentadas por células diferenciadas e células estaminais adultas (e os seus respectivos núcleos). A partir destas observações colocámos a hipótese de que, através da modulação da rigidez de MSCs, poderíamos aumentar a eficiência do processo de reprogramação usando um vector lentiviral que codifica factores de pluripotência.

O núcleo está mecanicamente acoplado a elementos do citoesqueleto através do complexo LINC (ligante do nucleoesqueleto ao citoesqueleto) e desta forma as forças mecânicas vindas da matriz extracelular podem ser transmitidas através do citoesqueleto até ao núcleo. Dependendo da rigidez do substrato, o núcleo está sob maior ou menor tensão, sendo eventualmente possível modular o seu módulo elástico se as células forem plaqueadas em plataformas com diferentes graus de rigidez.

Com este trabalho demonstrámos que ao plaquear MSCs em substratos com distintos graus de rigidez, é possível torná-las mais propícias a uma total reprogramação. Para além disto, verificou-se também um aumento na expressão de genes de pluripotência devido apenas ao facto de MSCs serem mantidas em cultura em substratos específicos. Ao analisarmos o estado de compactação da cromatina, bem como a área nuclear tornou-se evidente o efeito que a rigidez dos substratos tem

sobre as células. Assim sendo, os rácios do conteúdo eucromático e heterocromático dos núcleos, bem como a área nuclear mostraram ser modulados, o que sugere que os núcleos sofrem mecanomodulação. Foram também observadas diferenças no que ao tamanho das Adesões focais (FAs) e à organização das fibras de actina diz respeito.

Tomando em conjunto todos os nossos resultados, verificou-se que é possível melhorar o processo de reprogramação através da modulação da rigidez do substrato, e que o mecanismo por detrás desta melhoria pode estar intimamente relacionado com a mecanomodulação do núcleo.

Este progresso na geração de iPSCs humanas, recorrendo a substratos de rigidez definida, dá indícios de que os protocolos habituais de reprogramação celular em plataformas convencionais de cultura de células podem ser substancialmente melhorados plaqueando as células nesses mesmos substratos. Deste modo, aumentado a eficiência e a cinética da geração de células estaminais pluripotentes induzdas, estudos futuros poderão explorar a utilização de vectores de reprogramação não integrativos, considerados mais seguros para possíveis aplicações clínicas.

Palavras-chave: MSCs, mecanotransdução, iPSCs, eficiência de reprogramação

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	AF	Amniotic fluid
	AF-MSC	Amniotic fluid mesenchymal stem cells
	Alpha-MEM	Alpha modified Eagle's medium
	APTMS	Aminopropyltrimethoxisilane
B	bFGF	basic Fibroblast Growth Factor
	BM	Bone Marrow
	BM-MSCs	Bone marrow mesenchymal stem cells
	BSA	Bovine serum albumin
C	CD	Cluster of differentiation
	CFU-F	Colony forming unit-fibroblast capacity
D	DAPI	4',6-diamidino-2-phenylindole
	DMSO	Dimethyl sulfoxide
	DMEM	Dulbeco's modified Eagle's medium
E	*E*	Elastic modulus
	EBs	Embryoid Bodies
	ECCs	Embryonal carcinoma cells
	ECM	Extracellular matrix
	EGCs	Embryonic germ cells
	EpiSCs	Epiblast-derived stem cells
	ESCs	Embryonic stem cells
	EtOH	Ethanol
F	FAK	focal adhesion kinase
	FAs	Focal adhesions
	FBS	Fetal bovine serum
	FN	Fibronectin

	FP	Fluorescent protein
G	*G*	Shear modulus
H	hESCs	Human embryonic stem cells
	hiPSCs	Human induced pluripotent stem cells
	HLA-DR	Human leukocyte antigens disease resistance
	HLA-G6	Human leukocyte antigen G6
I	IFN-γ	Interferon gamma
	IL	Interleukine
	INM	Inner nuclear membrane
	iPSCs	Induced pluripotent stem cells
	IRES	Internal ribosomal entry site
K	KASH	Klarsicht, ANC-1, SyneHomology
	Klf4	Kruppel-like factor 4
	KO	Knock out
L	LINC	Linker of Nucleoskeleton to Cytoskeleton
M	MEFs	Mouse Embryonic Fibroblasts
	MFI	Mean Fluorescence Intencity
	mGSCs	multipotent germline stem cells
	MHC-class I	Major histocompatibility complex class I
	MSCs	Mesenchymal stem cells
	MW	Multi well
	MyoD	Myogenic differentiation
N	NEAA	Non essential amino acids
	nuc	Nuclear membrane-localized derivative
O	Oct4	Octamer-binding transcription factor 4

	ONM	Outer nuclear domain
P	PBB	PBS, BSA and (fetal) Bovine serum solution
	PBS	Phosphate buffered saline
	PDMS	Polydimethylsiloxane
	PFA	Paraformaldehyde
	PGCs	Primordial germ cells
	PRE	Post-transcriptional regulatory element
R	RN	Retronectin
	ROIs	Regions of interest
	RPTPα	Receptor-like protein tyrosine phosphatase
	RRE	Rev-responsive element
	RSV	Rous sarcoma virus U3 promoter
	RT	Room temperature
S	SA	Splice acceptor
	SCNT	Somatic-cell nuclear transfer
	SD	Splice donor
	SEM	Standard error of the Mean
	SFKs	Scr family kinases
	SFFV	spleen focus-forming virus promoter
	SH	Scr homology
	SSEA	Stage-specific embryonic antigen
	SUN	Sad1p, UNC-84
T	TCPs	Tissue culture polystyrene
	TGF-β	Transforming growth factor beta
	TRA	Keratan sulfate antigens
U	UC	Umbilical cord
	UCM	Umbilical cord matrix
V	VEGF	Vascular endothelial growth factor

	VASP	Vasodilator stimulating phosphoprotein
W	WJ	Wharton's jelly
	WJ-MSCs	Wharton's jelly mesenchymal stem cells

CHAPTER I

INTRODUCTION

I. INTRODUCTION

I.1. MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are, one type of adult stem cells, also called mesenchymal stromal cells (Prockop, 1997), mesenchymal progenitor cells or adult mesenchymal stem cells (Karahuseyinoglu et al., 2007). These cells are multipotent, able to self-renew and differentiate *in vitro* into several cell types within mesenchymal origin, namely adipose, osteogenic and chondrogenic lineages (Pittenger, 1999; Prockop, 1997). Beyond the mesenchymal lineages, MSCs were also reported to differentiate into several non-mesenchymal lineages, such as neural-like lineages (Karahuseyinoglu et al., 2007).

Friedenstein was the first to isolate MSCs from the bone marrow (BM), between 1960 and 1970, and characterize them as plastic-adherent and clonogenic cells with a fibroblast-like morphology and high ability to proliferate *in vitro*. These cells were characterize as clonogenic due to their colony forming unit-fibroblast (CFU-F) capacity, in other words they have the capacity to form colonies similar to fibroblasts (Beyer Nardi and da Silva Meirelles, 2006; Friedenstein et al., 1970; Pan et al., 2009).

I.2. SOURCES OF MESENCHYMAL STEM CELLS

Although MSCs were first identified and isolated from the BM (Friedenstein et al., 1970), several subsequent studies have shown that these cells can be isolated from other tissues such as adult mesenchymal tissues like adipose tissue, connective tissues of the dermis, skeletal muscle (Wagner et al., 2005), synovium, periosteum (Sakaguchi et al., 2005), dental pulp (Gronthos et al., 2000), and in fact any perivascular region of virtually any tissue (Crisan et al., 2008). Besides the adult tissues MSCs can also be isolated from extra-embryonic tissues like the umbilical cord matrix/Wharton's jelly (UCM/WJ), decidua and amniotic fluid (AF) (In 't Anker et al., 2004; Wang et al., 2004).

Extra-embryonic tissues constitute a good alternative source of MSCs, because these are more naïve and therefore have lower immunogenicity than MSCs from adult mesenchymal tissues (Malgieri et al., 2010). Furthermore the latter also decrease with

donor's age in terms of proliferative, expansion and differentiation capacity (Caplan, 2007).

Currently, the umbilical cord (UC) is often cryopreserved due to the potential clinical applications of MSCs present within that tissue. The collection of these cells is made by a simple, non-invasive, painless, and safe procedure, in contrast to BM aspiration. Therefore the UC raises no ethical or technical issues for use either for clinical purposes or scientific studies (Malgieri et al., 2010).

MSCs from the UC show a fibroblast-like morphology in culture and express typical MSC immunophenotypic markers. This type of MSCs does not express endothelial CD31 neither leukocyte surface markers, therefore MSCs from UC are classified as mesenchymal progenitors.

MSCs from the UC and from the Bone Marrow share most of the typical MSC immunophenotypic markers, although some secondary markers may differ (Table I.1). MSCs form both origin are positive for CD73, CD90 and CD105, and negative for CD34, CD45 and HLA-DR (Dominici et al., 2006). Nestin was also identified more recently as an important MSC marker (Mendez-Ferrer et al., 2010).

Table I.1: Surface markers expressed by MSCs from BM and from UCM /WJ. Adapted from (Anzalone et al., 2010)

Markers	*BM-MSC*	*WJC*
CD10	+	+
CD13	+	+
CD14	−	−
CD29	+	+
CD31	−	−
CD33	−	−
CD34	−	−
CD44	+	+
CD45	−	−
CD49e	+	+
CD51	+	+
CD54	+	NA
CD56	+	−
CD59	+	NA
CD68	NA	+
CD71	+	NA
CD73	+	+
CD79	−	NA
CD80	−	+
CD86	−	−
CD90	+	+
CD105	+	+
CD117	−	+
CD163	NA	−
CD166	+	+
CD235a	−	NA
CK-7	NA	−
CK-8	NA	+
CK-18	+	+
CK-19	+	+
Connexin-43	+	+
GATA-4	+	+
GATA-5	NA	+
GATA-6	NA	+
GFAP	+	+
HLA-A	+	+
HLA-B	+	+
HLA-C	+	+
HLA-DR	−	−
HLA-G	+	+
HNF-4α	NA	+
Nanog	+	+
Nestin	+	+
NSE	+	+
Oct3/4A	+	+
α-SMA	+	+
Vimentin	+	+

Abbreviation: NA, not applicable.

MSCs from extra-embryonic tissues have a close ontogenetic relationship with embryonic stem cells (ESCs), and because of that they have immunoprivileged characteristics (Chen et al., 2011), possess a wider multipotent plasticity (Lee et al., 2004) and proliferate faster than MSCs from adult tissues (Ki-Soo Park, 2006; Kim et al., 2013). For these reasons MSCs from neonatal tissues are very promising type of MSCs that may be used in scientific and clinical studies.

I.3. Mesenchymal stem cells from the Umbilical Cord Matrix/ Wharton's Jelly

The UC contains two arteries and one vein, which are surrounded by a gelatinous and connective tissue called Wharton's jelly, first described by Thomas Wharton in 1656 (Kim et al., 2013). The WJ is composed by a network of glycoprotein microfibrils, collagen fibers, and proteoglycans and contains fibroblast- and myofibroblast-like stromal cells (Malgieri et al., 2010; Mitchell et al., 2009).

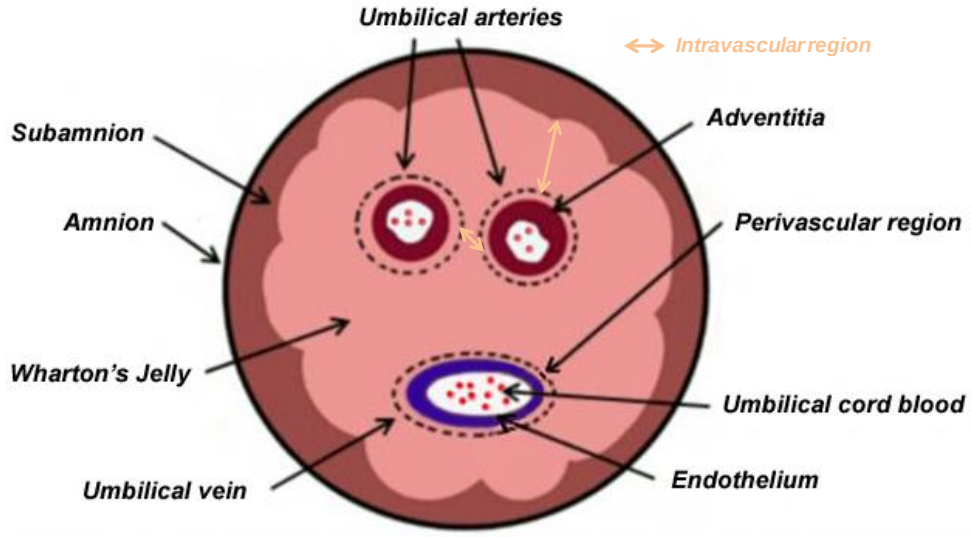

Figure I.1: Diagram shows anatomical compartments within the human umbilical cord. Five different regions have been found to contain MSCs: vein, the vein endothelium, perivascular region, subamnion and Wharton's jelly Adapted from (Kim et al., 2013)

McElreavey et al. reported for the first time the isolation of fibroblast-like cells from the WJ in 1991 (McElreavey et al., 1991). The MSCs from the Wharton's jelly (WJ-MSCs) can be isolated from three regions: the perivascular zone, the intravascular zone and also subamnion (Troyer and Weiss, 2008). These three regions have different properties, because there are significant differences in the number and nature of cells from the different regions (Karahuseyinoglu et al., 2007).

The mesenchymal features of WJ-MSCs have been confirmed by the expression of specific lineage cytoskeletal markers, such as SMA and vimentin (Conconi et al., 2011).

MSCs from WJ express matrix receptors (CD44, CD105, CD73, CD90),integrin markers (CD29, CD51) and also express significant levels of some MSCs markers (SH2, SH3, SH4) but not hematopoietic lineage markers (CD34, CD45) (Anzalone et al., 2010; Cao and Feng, 2009; Dominici et al., 2006; Wang et al., 2004). The most outstanding feature of WJ-MSCs is their unique ability to express the HLA-G6 isoform. This

molecule is implicated in immune-modulation, because it seems to play a role in the immune tolerance during pregnancy avoiding a maternal immune response against the fetus. Furthermore, WJ-MSCs also lack of CD86 which suggests that this type of MSCs is particularly appropriate for cell-based therapy (Conconi et al., 2011). Additionally, these cells must lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, and HLA-DR molecules are not expressed on MSC unless stimulated, for instance by IFN-γ (Dominici et al., 2006).

I.3.1. WHARTON'S JELLY MSCS AS AN APPROPRIATE SOURCE OF CELLS FOR REPROGRAMMING

Reprogramming efficiency is cell type-dependent and more research is needed to determine the best starting cell population of cells for iPSCs generation.

In this respect, mesenchymal stem cells are promising candidates with several advantages. MSCs can easily be isolated and expanded from various tissues, and comprise a multipotent subset of adult stem cells, which may be more suitable for reprogramming than differentiated cells. This hypothesis is supported by different studies that report the generation of iPSCs from Neural Stem Cells using just one or two factors (Hester et al., 2009; Kim et al., 2009; Kim et al., 2008). In 2011, by comparing MSCs with mature cells from the same lineage (osteo-progenitors), Niibe and colleagues (Niibe et al., 2011) concluded that immature tissue stem cells are more efficient cell source for reprogramming than partially committed ones.

In order to generate iPSCs from MSCs different sources were explored. Adipose tissue (Lister et al., 2011; Sugii et al., 2010), dental pulp (Oda et al., 2010; Yan et al., 2010), synovial tissue (Kim et al., 2011) , bone marrow (Ohnishi, 2012), umbilical cord and other extra-embryonic tissues are examples of that.

Among all the types of MSCs used for reprogramming, the most appealing are the ones from extra-embryonic tissues. These tissues are a source of vast amounts of cells that are poorly immunogenic and more naïve than MSCs from adult tissues (Malgieri et al., 2010). Furthermore the latter also decrease with donor's age in terms of proliferative, expansion and differentiation capacity (Caplan, 2007).

Moreover, MSCs from extra-embryonic tissues are likely exempt from incorporated mutations when compared with MSCs from adult tissues. Taking into account all the characteristics and of MSCs from extra-embryonic tissues, the MSCs from umbilical

cord matrix seems to be the most indicated cell type for the generation of induced pluripotent stem cells (iPSCs) (Cai et al., 2010; Niibe et al., 2011).

I.4. Reprogramming and Induced pluripotent stem cells (iPSCs)

During development, cells progressively lose potential and become more and more differentiated to accomplish the specialized functions of somatic tissues. For instance, zygotes and blastomeres of early morulas have the capacity to give rise to embryonic and extra-embryonic tissues, and are therefore called “totipotent”, whereas cells from the inner cell mass of the blastocyst can give rise to all embryonic but not all extra-embryonic tissues, and are called “pluripotent”. Cells, such as adult stem cells, present in adult tissues can only give rise to cell types within their lineage and are called either “multipotent” or “unipotent”, depending on the number of developmental options they have. Upon terminal differentiation, cells entirely lose their developmental potential (Stadtfeld and Hochedlinger, 2010).

In order to turn cells with less developmental potential into pluripotent cells (iPSCs) several studies were done over the last few decades. iPSC technology was established on the basis of numerous findings by past and current scientists in related fields. There were three major streams of research that turned possible the production of iPSCs.

In the 1950’s the researchers Briggs and King established the technique of somatic-cell nuclear transfer (SCNT), or “cloning” (Briggs and King, 1952), working on a frog, *Rana pipiens*, became the first to successfully transplant living nuclei in multicellular organisms. They transplanted blastula nuclei into enucleated eggs, which then developed into normal embryos.

In 1962, John Gurdon reported that his laboratory had generated tadpoles from unfertilized eggs that had received a nucleus from the intestinal cells of adult frogs (Gurdon, 1962). The logical consequence of Gurdon's success, that the nuclei of differentiated cells retain their totipotency potential, provided a key conceptual advance in developmental and stem cell biology. More than three decades later, Ian Wilmut and colleagues reported the birth of Dolly, the first mammal generated by somatic cloning of mammary epithelial cells by nuclear transfer (Wilmut et al., 1997).

While SCNT is a powerful tool to probe the developmental potential of a cell, it is technically challenging and not well suited for genetic and biochemical studies.

Moreover, for human studies, several technical and ethical issues arise, namely those related to the availability of human eggs.

The second stream was the discovery of "master" transcription factors. In 1987, a Drosophila transcription factor, Antennapedia, was shown to induce the formation of legs instead of antennae when ectopically expressed (Schneuwly et al., 1987). In the same year, a mammalian transcription factor, MyoD, was shown to convert fibroblasts into myocytes (Davis et al., 1987). These results led to the concept of a "master regulator", a transcription factor that determines and induces the fate of a given lineage.

Another major advance towards generating iPSCs was the establishment of immortal pluripotent cell lines from teratocarcinomas, tumors of germ cell origin. These cell lines were called embryonal carcinoma cells - ECCs - and keep pluripotency when clonally expanded in culture (Kahan and Ephrussi, 1970; Kleinsmith and Pierce, 1964).

Pluripotent cell lines have also been derived from other embryonic and adult tissues upon explantation in culture. For instance, epiblast-derived stem cells - EpiSCs - (Tesar et al., 2007) have been isolated from post-implantation embryos; embryonic germ cells (EGCs) (Matsui et al., 1992) have been derived from primordial germ cells - PGCs - of the midgestation embryo, and multipotent germline stem cells - mGSCs - (Kanatsu-Shinohara et al., 2004; Ko et al., 2009) have been generated from explanted neonatal and adult testicular cells. A common molecular characteristic among these cell types is the expression of endogenous Oct4, which may therefore serve as an important marker for whether or not a cell can give rise to pluripotent cell lines (Stadtfeld and Hochedlinger, 2010).

The third principle that contributed to the discovery of induced pluripotency was the observation that lineage associated transcription factors, which help to establish and maintain cellular identity during development by driving the expression of cell type-specific genes while suppressing lineage-inappropriate genes, can change cell fate when ectopically expressed in certain heterologous cells (Smith et al., 1988; Thomson et al., 1998).

In 2000, Yamanaka and colleagues began testing the idea that factors that maintain pluripotency in ESCs might induce pluripotency in somatic cells (Yamanaka, 2009). In

2006, Takahashi and Yamanaka came with a groundbreaking report in the stem cell field showing that the forced expression of only four transcription factors (Oct4, Sox2, Klf4, and c-Myc) among a list of 24 pluripotency-associated candidate genes, delivered by viral transfection, was sufficient to convert fibroblasts into ESC-like cells. The cells were named induced pluripotent stem cells (iPSCs) and this process was called reprogramming (Takahashi and Yamanaka, 2006; Yamanaka, 2009).

Induced Pluripotent Stem Cells are similar to ESC in their morphology, expression of important pluripotency marker genes, and their ability to form teratomas (tumours comprised of diverse tissue types) when injected subcutaneously into immune-compromised mice (Takahashi and Yamanaka, 2006; Yamanaka, 2009).

However, the "first generation" of iPSCs had different global gene expression patterns, expressed lower levels of several key pluripotency genes compared with ESCs, showed incomplete promoter demethylation of ESCs regulators such as Oct4, and failed to generate postnatal chimeras or contribute to the germline. These characteristics indicated that the iPSCs were not fully reprogrammed (Takahashi and Yamanaka, 2006). Nevertheless, by modification of the induction protocols, Yamanaka's laboratory and others generated iPSCs fully reprogrammed. For instance by selecting for the reactivation of the essential pluripotency genes Nanog or Oct4 instead of Fbxo15, the generated iPSCs were molecularly and functionally more similar to ESCs. More recently, rare iPSC lines have been identified as being capable of generating "all-iPSC" mice upon injection into tetraploid blastocysts, suggesting that at least some iPSC clones have a developmental potency equivalent to ESCs (Boland et al., 2009; Kang et al., 2009).

In 2007, Yamanaka and colleagues (Takahashi et al., 2007) reprogrammed human fibroblasts using the same four factors that were discovered for mice in 2006 (Takahashi and Yamanaka, 2006), using retroviruses to deliver and express the genes.

Since these outstanding reports, iPSCs lines have been generated from human (hiPSCs), monkey and rat somatic sources, as well as from multiple somatic cell types, such as keratinocytes, neural cells, stomach and liver cells, melanocytes, genetically labeled pancreatic β-cells and terminally differentiated lymphocytes, using various vehicles to deliver the factors into target cells (Colman and Dreesen, 2009; Stadtfeld and Hochedlinger, 2010).

I.4.1. FACTOR DELIVERY INTO TARGET CELLS

The reprogramming process is highly inefficient (Maherali et al., 2008; Takahashi and Yamanaka, 2006). Due to the requirement of high expression levels of the transgenes for successful reprogramming, the original virally generated iPSC lines contained a large number of random viral integrations into the genome. Obviously, the integration of exogenous genome represents a high risk of random mutagenesis and tumor development. Therefore, much of the technical developments and different approaches have been designed, following the original viral delivery, to shuttle reprogramming factors into target cells (Figure I.2). These have been focusing on eliminating or avoiding transgene integrations as well as increase the efficiency of the reprogramming process and the quality of resultant iPSCs (Hussein and Nagy, 2012; Stadtfeld and Hochedlinger, 2010).

Depending on cell type, vectors, reprogramming mix and infection protocols, reprogramming efficiency range from 0.001 to 40% (Anokye-Danso et al., 2011; Eminli S, 2009; Hong et al., 2009; Maherali et al., 2008). More recently, there was reported the reprogramming of somatic cells with near to 100% efficiency (Rais et al., 2013).However, so high efficient is always questionable because, usually there are a lot genetic manipulation intrinsic and the method is not so reproducible.

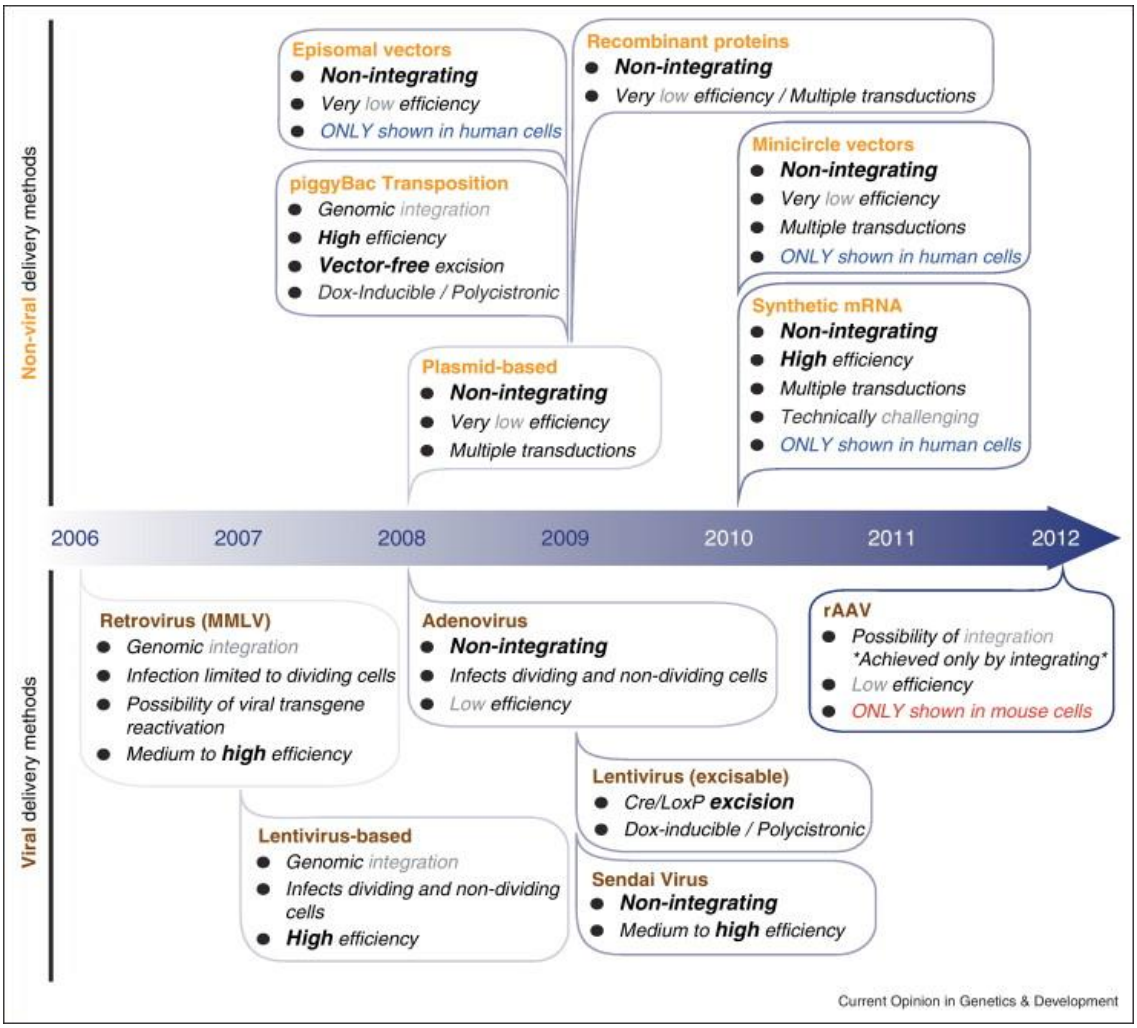

Figure I.2: Factor delivery methods for iPSCs generation. Over the years much research has been done in order to improve the delivery and expression of reprogramming factors and also to avoid issues associated to integration of viral genome in target cells. The delivery methods are divided in two different groups; (i) non-viral and (ii) viral methods. The first are mostly non-integrative methods, avoiding some risks associated with integration of exogenous genome; however these methods present low efficiency. On the other hand, viral methods present higher efficiency, but are mostly integrative vectors. Adapted from (Hussein and Nagy, 2012)

I.4.2. LENTIVIRAL VECTORS

Lentiviral vectors have the ability to carry large transgenes (~ 8 kb) and to efficiently infect and integrate these genes into the genomes of both dividing and non-dividing cells. These characteristics make them ideal candidates for transport of genetic material into cells and tissues.

Lentiviruses are members of the *Retroviridae* family and their characteristics make them particularly useful for applications requiring the expression of large or multicistronic transgenes in target cells (Semple-Rowland and Berry, 2013).

Warlich and colleagues (Warlich et al., 2011) developed fluorescence-coded lentiviral vectors that initially trigger high-level expression of the reprogramming factors and are subsequently rapidly silenced in reprogramming cells.

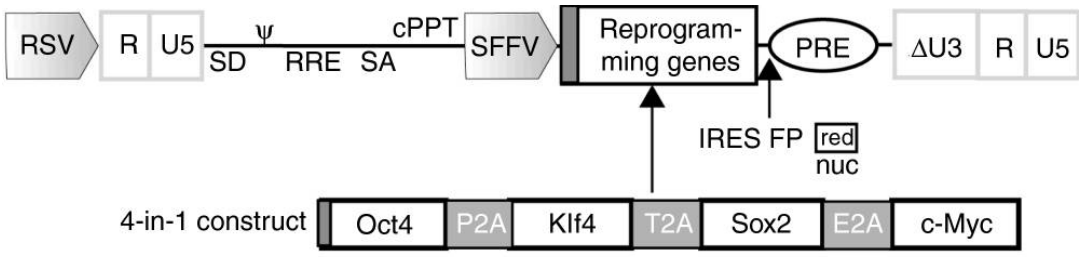

Figure I.3: Multicistronic reprogramming vector all-in-one self-inactivating vector expressing four reprogramming factors. FP, fluorescent protein (red: dTomato); IRES, internal ribosomal entry site; nuc, nuclear membrane-localized derivative; PRE, post-transcriptional regulatory element; RRE, rev-responsive element; RSV, Rous sarcoma virus U3 promoter; SA, splice acceptor; SD, splice donor; SFFV, spleen focus-forming virus promoter; ψ, packaging signal. Adapted from (Warlich et al., 2011)

The lentiviral vector depicted in Figure I.3 encodes murine or human versions of the canonical reprogramming factors (Oct4, Klf4, Sox2, c-Myc). The vector allows efficient co-expression of reprogramming factors and a fluorescent marker (dTomato) on the same mRNA to monitor the expression of the reprogramming factors. This is a combinatorial vector that coexpresses Oct4, Klf4, Sox2, and c-Myc (4-in-1 vector).

In addition, this vector has a number of modifications to the expression cassette that improve reprogramming factors and fluorescent marker expression, because conversion to pluripotency requires robust reprogramming factors expression and a clear fluorescence signal is a prerequisite for imaging/cell tracking studies (Warlich et al., 2011).

Besides the particularity of being a 4-in-1 vector and having a fluorescent marker, this vector also has a transcription promoter that facilitates the binding of viral reprogramming factors to the pluripotency genes of the cell, by opening the chromatin. So, when cells are infected they show a red fluorescence signal (dTomato), indicating that reprogramming factors within the vector are being expressed. This vector is also constituted by a post-transcriptional regulatory element that induces some epigenetic remodeling, which silence the vector. Therefore, red fluorescence is shut down, indicating that cells are possibly reprogrammed (Warlich et al., 2011).

In summary, this vector constitutes a system in which the onset and silencing of reprogramming factor expression can be monitored with high sensitivity, and which shows a remarkable frequency of reprogramming cassette silencing in reprogramming cells especially when using a cassette for combinatorial expression of reprogramming factors (Warlich et al., 2011).

I.4.3. PLURIPOTENT STEM CELLS AND THEIR PHYSICAL PLASTICITY

It is known that pluripotent stem cells do not express lamin A, and that this nuclear protein is increasingly expressed along differentiation into most cell types. Absence of lamin A in ESCs and strong expression of lamin A in the nuclear envelope of somatic cells may allow to distinguish pluripotent stem cells from differentiated cells (Zuo et al., 2012). Furthermore it was proved that the absence of lamin A keeps the cells in an undifferentiated state and accelerates the reprogramming process (Zuo et al., 2012).

Within this topic of physical plasticity of the cells, there are several studies indicating that the stemness of cells is associated with their stiffness and physical plasticity. Pajerowski showed in 2007 that physical plasticity of the nucleus decreases with the differentiation process, thus pluripotent stem cells have more compliant nuclei than multipotent cells, and these are both more compliant than nuclei from completely differentiated cells (Figure I.4) (Pajerowski et al., 2007). These observations were later confirmed by other studies (Mazumder and Shivashankar, 2010).

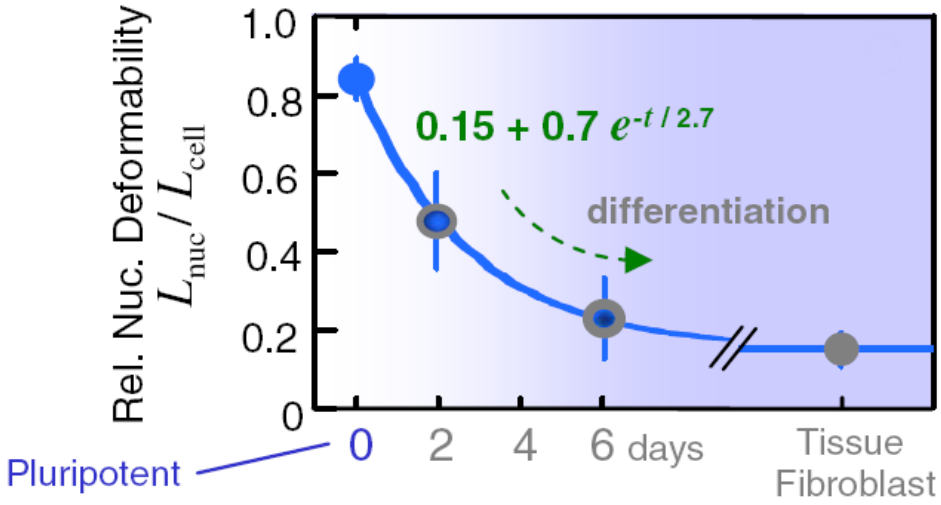

Figure I.4: Variation of nuclei physical plasticity with the differentiation process. Nuclei of differentiated cells are less deformable than the nuclei of stem cells. As well the cells became more differentiated as the plasticity of their nuclei decreases sharply. Adapted from (Pajerowski et al., 2007)

Moreover, Hammerick reported in 2011 that once a cell is reprogrammed, its overall stiffness decreases to levels characteristic of ESCs (Hammerick et al., 2011).

There seems to be a connection between cell stemness and mechanical forces within the cell that could be somehow important and beneficial to the efficiency of cell reprogramming process (Downing et al., 2013; Lai et al., 2010; Zhang and Kilian, 2013). Hence, the mechanobiology field may provide some clues about the influence of mechanical stimuli on cell pluripotency maintenance and induction.

I.5. MECHANOTRANSDUCTION

In the context of intracellular signaling cells are sensitive not only to soluble factors, but also to ligands present in the extracellular matrix (ECM), such as ECM proteins. Moreover, cells respond to extracellular environment stimuli not only on a basis of the biochemical nature of ligands, but also to elements intrinsic to the extracellular environment such as rigidity, the geometry, local curvature of the matrix, and to the applied stress or strain using cell surface receptors (Figure I.5) (Shivashankar, 2011).

Mechanotransduction is by definition the mechanism by which cells transform mechanical signals into biochemical responses (Ingber, 2006). Cells sense and respond to mechanical signals translating that information into biochemical and morphological changes and eventually lineage or fate specification. At the molecular level, matrix

sensing requires the ability to pull against the matrix and also cellular mechanotransducers to generate signals based on the force that the cells must generate to deform the extracellular matrix (Engler et al., 2006). Numerous molecules and subcellular structures have been shown to mediate force sensing and mechanochemical conversion.

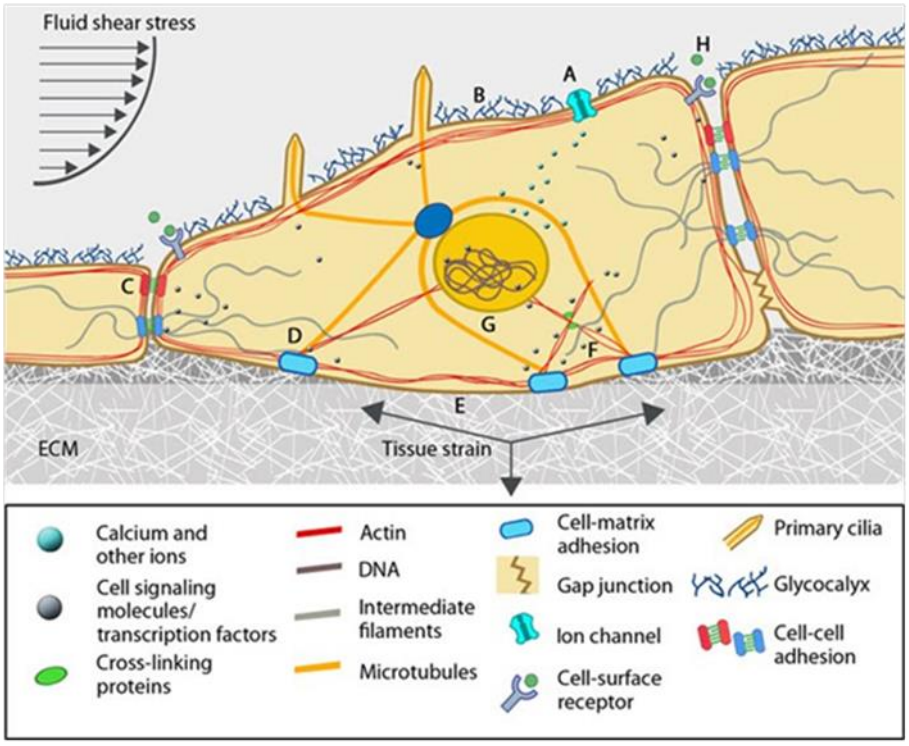

Figure I.5: Mediators of cellular mechanotransduction. There are several molecules, cellular components and extracellular structures that contribute to mechanotransduction. Some of them are ECM, cell-extracellular matrix, cell-cell adhesions, membrane components, cytoskeletal filaments and some nuclear structures. (A) In response to lipid fluidity and stretching of the plasma membrane ion channels are activated in order to allow ion flow. (B) The glycocalyx has the ability to sense fluid shear stress. (C) Cell-cell adhesion complexes: Adherens junctions (red) and desmosomes (light blue) communicate with cytoskeleton, while gap junctions make the contact between the cytoplasm of adjacent cells. (D) Cell-matrix adhesion complexes are responsible to sense and signal the changes in the extracellular environment. (E) Force-induced conformational changes can initiate mechanotransduction signals. (F) Intracellular strain could change protein conformations improving their capacity to bind cytoskeletal components. (G) The nucleus sense the mechanical signals responsible to modulate transcription. (H) Changes in inter-cellular space are cell-type specific. Different cell types have different concentration of signaling molecules and proteins that bind cell-surface receptors. Adapted from (Mechanobiology Institute, 2012)

Within all these structures that drive mechanotransduction responses, maybe the most well described are primary cilia, stretch-modulated ion channels and focal adhesions. However, other sites for mechanosensing such as nuclear lamina and the nucleus itself, the cytoskeleton and the cortical membrane are equally very important and crucial for mechanotransduction (Chen, 2008).

More recently the mechanobiology field has begun to recognize that stem cell microenvironments present specific biophysical cues that strongly influence stem cell behavior regulating a variety of cellular functions such as migration, proliferation, differentiation and maintenance of multipotency (Engler et al., 2006; Fu et al., 2010; Lee et al., 2013; Lu et al., 2014; McBeath R, 2004; Mih et al., 2012; Sun et al., 2012; Zhang and Kilian, 2013). For example, stiffness varies widely between different tissues and within individual tissues, resulting in a diversity of mechanical signals sensed by the cells which may be an important component of the stem cell niche (Keung et al., 2010).

I.5.1. STIFFNESS

The stiffness of materials is measured by the relationship between applied forces and the resulting stretch of a material. In a biological context, a material's stiffness or elasticity is referred to as Elastic (or Young's) modulus *E* that consists of the amount of force per unit area needed to deform the material by a given fractional amount without any permanent deformation, being a high elastic modulus corresponding to high stiffness and low deformability (Keung et al., 2010).To quantify it, the Young's modulus and the shear modulus are commonly employed. For the elastic modulus *E*, the force is applied perpendicular to the material's surface, whereas for shear modulus *G*, the force is applied parallel to the surface (Figure I.6). Both elastic and shear moduli represent the amount of force per unit of area. In the case of the elastic modulus is the force required to double the length of a material, and regarding the shear modulus is the force required to deflect it by a distance equal to its height. These moduli are related by the following function: $E = 2G(1+\boldsymbol{\nu})$, where $\boldsymbol{\nu}$ is the Poisson ratio. For materials that do not change volume under stretch, the Poisson ratio equals 0.5. As a consequence, the elastic modulus will be three times its shear modulus. The units for rigidity are force per area, with the SI unit being the Pascal (Moore et al., 2010).

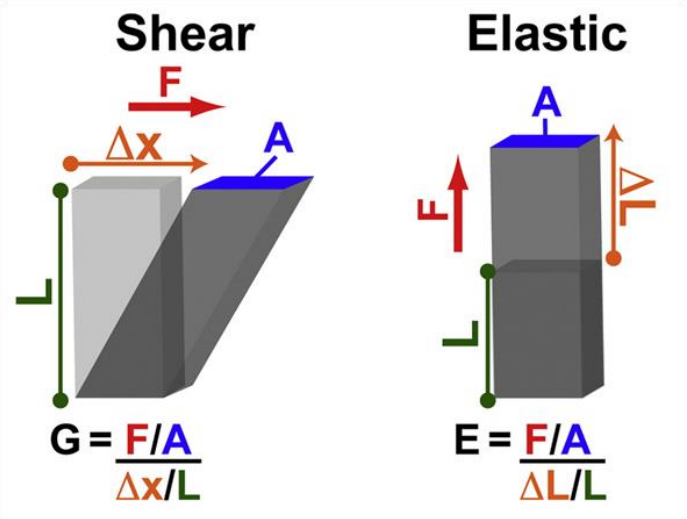

Figure I.6: Rigidity moduli. The amount of force applied per area (F/A) is the stress, and strain is the displacement in the direction of applied force relative to initial length (Dx/L or DL/L). While both elastic (*E*) and shear (*G*) moduli represent the ratio of stress over strain, the difference is in the direction of the applied force. Adapted from (Moore et al., 2010)

Cells generally show better *in vitro* behavior when cultured on materials with stiffness similar to that of their native microenvironmet, so the rigidity preferences of cells generally reflect their native environments (Moore et al., 2010). The linear elastic modulus of adult mammalian tissues spans from < 1 kPa (i.e., 1000 N/m2, or 1 nN/ μm^2 for units more relevant to cell biology systems) for brain, to 10 MPa for bone.

Table I.2: Linear Elastic Modulus or Stiffness of Mammalian Tissues. Adapted from (Keung et al., 2010)

Tissue	Stiffness/Elastic Modulus (Pa)
Fat	17
Mammary Gland	167
Brain	137-786
Liver	640
Kidney	7,500
Skeletal muscle	12,000
Cartilage	949,000
Bone	4-400 x 10^6

I.5.2. MECHANISMS OF MECHANOTRANSDUCTION AT THE CELL-ECM INTERFACE

Cellular behavior is influenced by mechanical stimuli originating at the interface between cells and the extracellular matrix. The origin of the force or stress that initiates the signal may be either at the cell, the ECM, or both. Although considerable

advances have been made in the past years, the mechanisms by which cells sense stiffness and other biophysical aspects of the extracellular environment remain poorly understood (Moore 2010, Eyckmans 2011).

It is generally believed that mechanotransduction initiates at focal adhesions (FAs), cell membrane regions that are essential for cellular attachment to ECM elements. These regions become highly enriched in integrins, transmembrane receptor proteins that - on their extracellular domain - bind to ECM proteins such as fibronectin, collagen or laminin. In turn, the intracellular domains of integrins recruit adapter molecules such us talin, paxilin and vinculin which allows the biding of focal adhesion kinase (FAK) forming the structure called focal adhesion. FAK does not bind to integrins directly, but its tyrosine kinase activity increases with mechanical force and decreases without it (Moore et al., 2010). In addition to these proteins, zyxin, VASP (Vasodilator stimulating phosphoprotein) and α-actin are also recruited to the FA, allowing the binding of the actin cytoskeleton (FigureI.7) (Eyckmans et al., 2011).

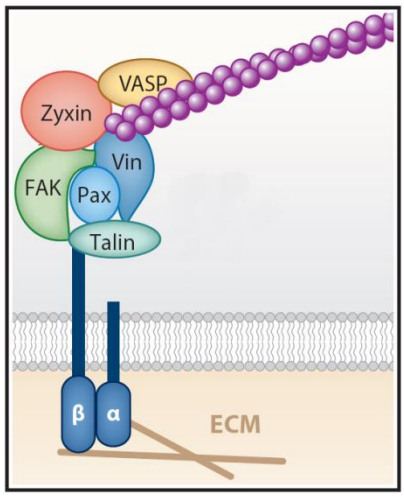

Figure I.7: Focal adhesion organization. Schematic representation of focal adhesion structure that includes transmembrane heterodimeric integrin, talin, paxilin (Pax), vinculin (vin), focal adhesion kinase (FAK), zyxin and vasodilator-stimulated phosphoprotein (VASP), and the actin cytoskeleton (in purple) bonded to the FA. Adapted from (Sun et al., 2012)

In fact, mechanotransduction can occur by two different ways. FAs perform a physical-anchoring function, as well as biochemical signaling. Once the FAK is activated, it could activate the MAP kinase and phosphatidylinositol 3-kinase signaling pathways regulating diverse cell functions, such as migration, differentiation, and proliferation (Sun et al., 2012).

On the other hand, FAK could also activate the RhoA that is a key molecular regulator of actin cytoskeleton tension. Once RhoA is activated, it will recruit myosin II to bind actin cytoskeleton. This motor protein will increase cytoskeleton tension leading to a reinforcement of FAs (Figure I.8) (Eyckmans et al., 2011). RhoA activity increases with mechanical force and decreases without it, which suggest that stiffness alters directly the degree of myosin activation and cellular contractility itself (Chen, 2008).

In turn, actomyosin cytoskeleton transmits the tension to the nucleus through LINC complex, regulating several cell functions in a much faster and direct way (Ning Wang, 2009). The mechanosignaling through LINC complex will be explained more in detail in section I.5.3.

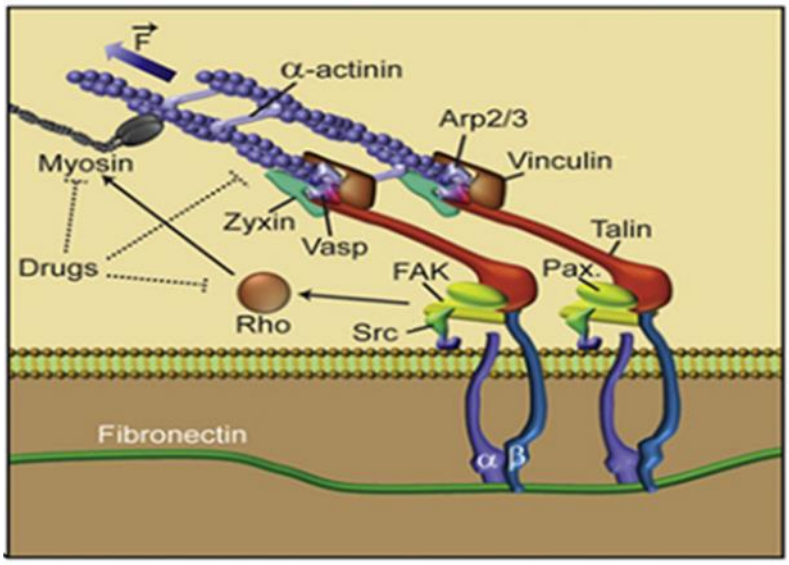

Figure I.8: Mechanotransduction at the focal adhesion. After the formation of the focal adhesion, RhoA is activated by FAK and recruits myosin to bind actin cytoskeleton, increasing the cytoskeleton tension and leading to the focal adhesion reinforcement. Adapted from (Eyckmans et al., 2011)

Taking these evidences and other observations into account, one mechanism was designed and proposed to explain rigidity sensing by cells. Integrins bind to ECM proteins and FAs initiate. Next, cells exert force on the substrate through actomyosin contraction, resulting in retrograde movement of actin fibers. Depending on the extent of such movement, which in turn depends on the rigidity of the substrate, talin may become stretched or not. In very soft substrates, the matrix deforms in response to the force exerted by the cell and talin does not stretch (Figure I.9 bottom left).

Otherwise, on stiffer substrates, the matrix does not deform and then, talin or other mechanosensitive adapter proteins stretches under actomyosin-induced tension enhancing its scaffold potential by revealing unexposed domains that will interact with other signaling proteins (Figure I.9 bottom rigth). As a result, stretching of talin reinforces focal adhesions by recruiting vinculin and other FA proteins (Moore et al., 2010).

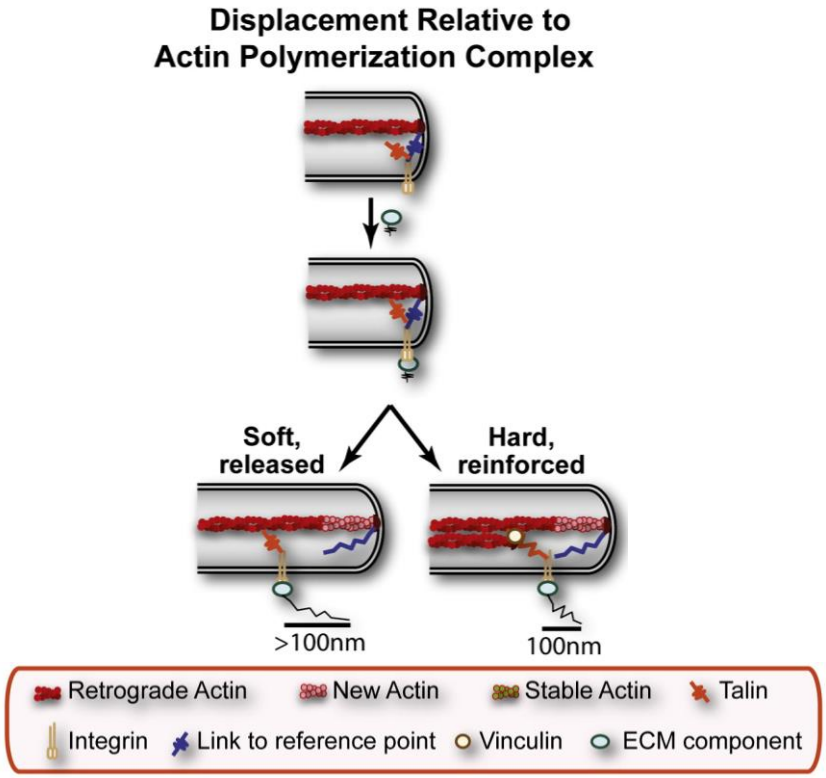

Figure I.9: Proposed model for rigidity sensing by cells and FAs reinforcement. As reference structure this model has the polymerization complex, and the key decision is based on whether the extension of the link to retrograde flowing actin (e.g. talin) occurs before the link to the reference structure is broken. Adapted from (Moore et al., 2010)

I.5.3. MECHANOSIGNALING THROUGH THE CELL TO THE NUCLEUS

Actin, a major constituent of the cytoskeleton, can form filaments which are prone to the tension forces applied by motor proteins like myosin. Myosin produces force and displacement through interaction with actin filaments (resembling a motor and cable system), forming a complex often referred to as actomyosin.

Actomyosin cytoskeleton has an important role in mechanotransduction events; it connects multiple parts of the cell membrane as well as the cell membrane to the nucleus. At the cell membrane, actomyosin filaments anchor into clusters of proteins

that constitute focal adhesions (FAs), which link the cytoskeleton through transmembrane integrins to the ECM. Applying force to this cell-ECM unit leads to structural deformations and rearrangements of the ECM and force transmission through the FA, as mentioned before, and leads also (given the highly interconnected nature of the cytoskeleton) to deformation of several aspects of intracellular structures, including the position of mitochondria, endoplasmic reticulum, and the nucleus (Eyckmans et al., 2011).

As it is known, the cytoskeleton is a critical component of cellular morphology, but emerging evidence suggests that it may also have important consequences for maintenance of functional nuclear architecture and its mechanical properties (Shivashankar, 2011).

Although the intracellular mechanisms of mechanotransduction are still extremely difficult to demonstrate, it has been postulated that force-induced nuclear deformations are potential regulators of genomic structure and accessibility of transcription factors to specific genetic targets (Iyer et al., 2012; Mazumder and Shivashankar, 2010).

The nucleus is mechanically coupled to cytoskeletal elements by the LINC (Linker of Nucleoskeleton to Cytoskeleton) complex through contacts across the nuclear envelope (Figure I.10). So the cytoskeleton forces are transmitted to the nucleus through this complex, modulating the nuclear shape, size and consequently the genomic structure (Lovett et al., 2013; Mazumder and Shivashankar, 2010).

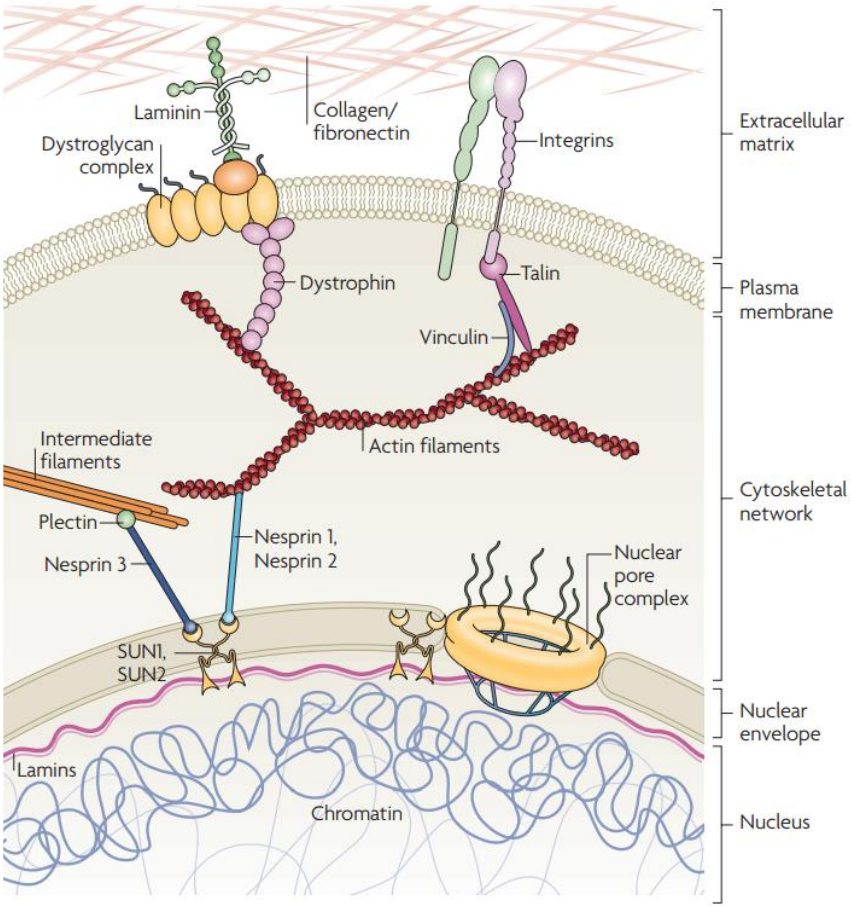

Figure I.10: Force transmission between the ECM and the nucleus. Extracellular forces are transmitted through integrins that are linked to some protein of the extracellular matrix (ECM) forming the focal adhesions (FAs) on the intracellular domain. FAs recruit the actin cytoskeleton and transmit the extracellular signaling through the cytoskeleton network. The actin cytoskeleton is coupled to the nucleus through nesprins (isoforms 1 and 2). These proteins interact with inner nuclear membrane proteins (SUN1 and SUN2) that in turn interact with nuclear envelope proteins such as lamins and emerin. Lamins form stable nuclear structures that can bind to DNA transmitting the forces from ECM to the nuclear interior. Adapted from (Jaalouk and Lammerding, 2009)

Thus, mechanical forces from the ECM can be transmitted to the nuclear surface through the cytoskeleton, while the LINC complex constitutes a physical bridge between the nuclear lamina and the cytoskeleton (Figure I.11) (Jaalouk and Lammerding, 2009; Mejat and Misteli, 2010).

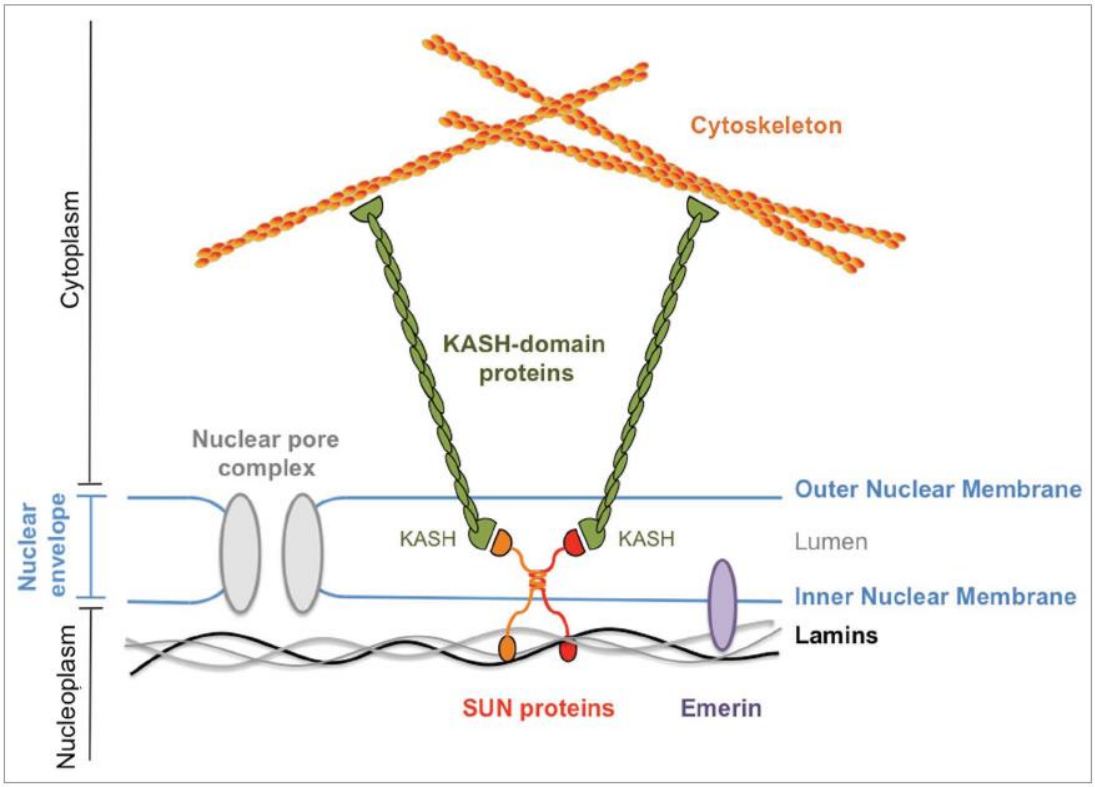

Figure I.11: Representation of the LINC complex. The LINC complex contains four general components. At the INM (inner nuclear membrane) is a SUN-domain transmembrane protein and in the ONM (outer nuclear membrane) is a KASH-domain protein which physically interacts with each other in the lumen of the nuclear envelope. SUN-domain proteins interact with the lamina and INM-associated proteins on the nucleoplasmic side. On the cytoplasmic side, the KASH-domain protein is in contact with the cytoskeleton. (Mejat and Misteli, 2010).

Knowing that cytoskeleton is able to transmit the information of mechanical signals to the nucleus through the LINC complex, Lovett and colleagues recently reported that the nuclear shape is modulated by substrate rigidity in the same way the whole cell is. Namely, the nucleus becomes more elongated and displays decreased height in response to stiffer substrates, in contrast to a more round shape when in culture on softer substrates. These results suggest that when cells are cultured on low stiffness conditions, nuclei become more relaxed, while under high stiffness conditions, nuclei seem to be stretched, somehow displaying lower physical plasticity (Lovett et al., 2013).

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