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# Combination of nano and microcarriers for stem cell therapy of Huntington's disease: new regenerative medicine strategy

Emilie André

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# Thèse de Doctorat

**Emilie ANDRE**

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Doctor degree from the University of Angers and from the University  
of Santiago de Compostela*

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Technology**

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## **Combination of nano and microcarriers for stem cell therapy of Huntington's disease: new regenerative medicine strategy**

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"La science a fait de nous des Dieux, avant même que nous soyons dignes d'être des  
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## ABBREVIATIONS

### **A**

AP: aminated pullulan

### **B**

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

### **C**

cryo-TEM: cryo- transmission electron microscopy

CNS: central nervous system

CNTF: ciliary neurotrophic factor

### **D**

DARPP32: dopamine and cAMP regulated phosphoprotein 32

DMEM: Dulbecco's modified eagle's Medium

DMSO: dimethyl sulfoxide

DOPE: 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine

DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane

### **E**

EDTA: ethylenediaminetetraacetic acid

EGF: ephthileium growth factor

ELISA: enzyme-linked immunosorbent assay

ESC: embryonic stem cells

### **F**

FDA: food and drug administration

bFGF: basic fibroblast growth factor

### **G**

GABA: gamma-aminobutyric acid

GAD65: glutamic acid decarboxylase 67

GDNF: glial cell line-derived neurotrophic factor

GF: growth factor

GRAS: generally recognized as safe

### **H**

HD: Huntington's disease

HTT: Huntingtin gene

htt: protein huntingtin

hMSC: human mesenchymal stem cells

### **I**

IA: ibotenic acid

iPS: induced pluripotent stem cells

iRNA: interferent RNA

### **K**

KA: kainic acid

### **L**

LGE: lateral ganglion emiscence

LNC: lipids nanocaspules

### **M**

MIAMI: Marrow-isolated adult multilineage inducible cells

mhtt: mutant of the protein huntingtin

MSC: mesenchymal stem cell

MSN: medium spiny neurons

### **N**

NCs: nanocarriers

NMDA: N-methyl-D-aspartate

NMR: nuclear magnetic resonance

NP: Nanoparticles

### **P**

P188: poloxamer 188

PA: poly-arginine

PAM: phamacologically active microcarriers

PBS: phosphate buffer saline

PD: Parkinson's disease

PEG: polyethylene glycol

PFA: paraformaldehyde

PLGA: poly(lactic-co-glycolic acid)

## Q

QA: quinoilinic acid

qPCR: polymerase chain reaction

## R

REST/NRSF: RE1-silencing  
transcription factor/neuron-restrictive silencer  
factor

RNA: ribonucleic acid

RNAi: interference RNA

RT: reverse transcription

## S

SEM: scanning electron microscopy

siRNA: small interfering RNA

Shh: Sonic hedgehog

SP: solid span nanoparticles

## T

TEM: transmission electron  
microscopy

## V

VPA: valproic acid

## W

W: water

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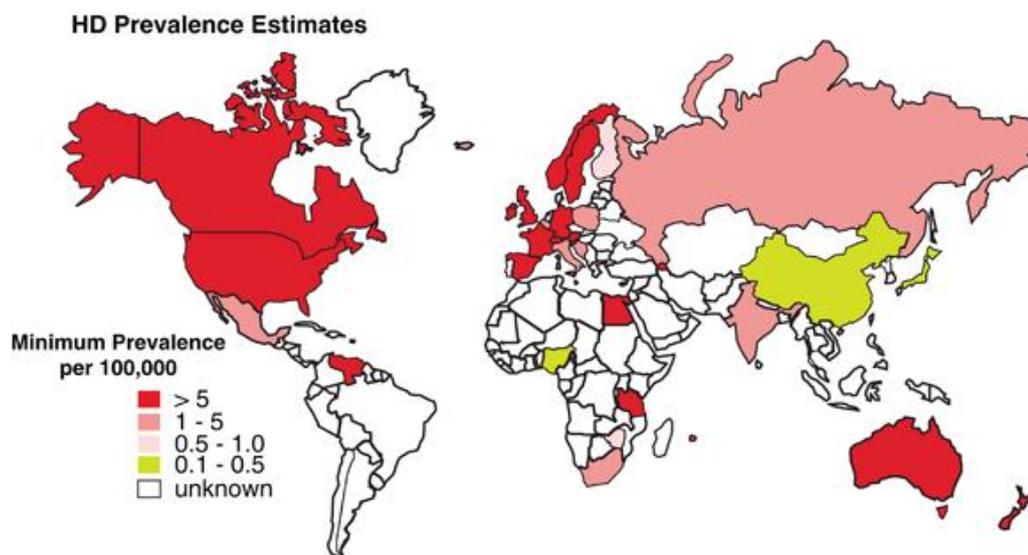


# INTRODUCTION

This PhD thesis has as main objective to offer an innovative tissue engineering strategy for Huntington's disease by combining nanocarriers delivering siRNA in mesenchymal stem cells and microcarriers releasing therapeutic proteins. This project is part of a general strategy of the laboratory INSERM U1066 "Biomimetic Micro and nanomedicine" from Angers and the department "Pharmacy and Pharmaceutical Technology" from Santiago de Compostela proposing an alternative and safe treatment in neurodegenerative disorders.

## 1. HUNTINGTON'S DISEASE.

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder with prevalence in Europe of about 10 per 100,000 births (**Figure 1.**) [1, 2]. HD appears in mid-life leading to death 15-20 years later and involves the triad signs and symptoms: involuntary movement disorders called Huntington's chorea, cognitive impairment and psychiatric manifestations.



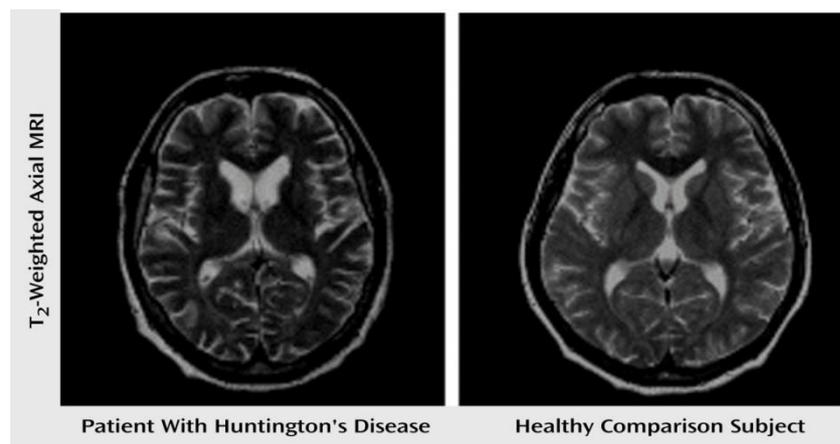
**Figure 1.** Worldwide estimates of the prevalence of HD. Overall, the prevalence of HD is much higher in European populations than in East Asia [3].

HD is one of the nine neurodegenerative disorders caused by the expansion of cytosine-adenine-guanine (CAG) triplet repeat sequence [4].

This disorder is characterized by an unstable repetition of triplet cytosine-adenine-guanine (CAG) of the Huntingtin (*htt*) gene in exon 1 of chromosome 4. It is translated at the protein level by a polyglutamine expansion at the NH<sub>2</sub>-terminal part of the protein huntingtin (HTT) [5]. The *htt* gene is considered as normal when it contains less than 27 CAG repeats and generally more than 40 repeats defines the adult-onset HD. The age of HD onset is inversely correlated with the length of the expansion, with variable age-dependent penetrance between 36 and 39 CAG repeats, but full penetrance at 40 or more repeats. In other words, people with 36-39 CAG repeats are at a risk of developing all the HD symptoms [6] and conversely, a larger number of repeats is usually associated with an earlier onset of signs and symptoms [7].

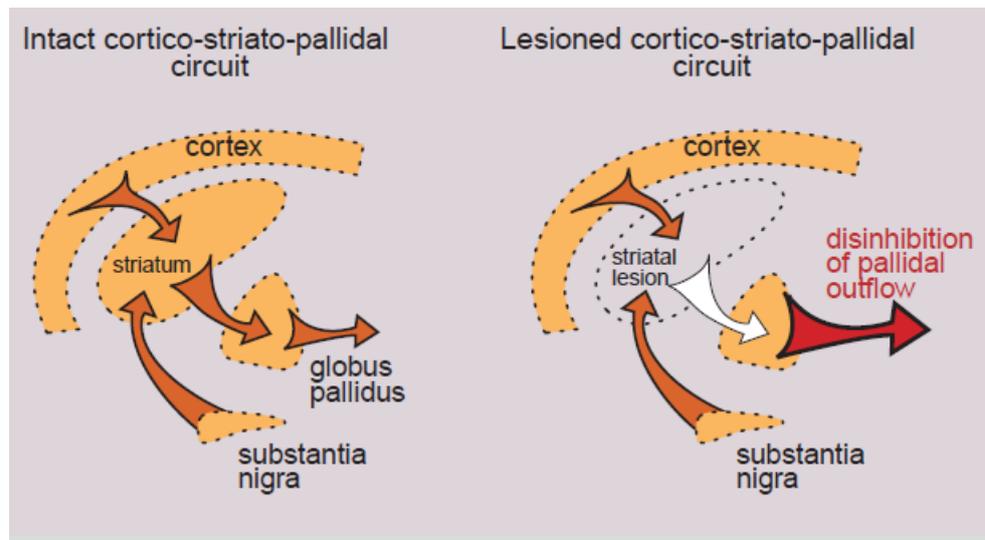
The HTT protein has ubiquitous roles in apoptosis [8], regulating microtubule-based transport [9] and scaffolding of cytoskeletal molecules at synapses [10]. Therefore, mutant HTT (mHTT) primarily affects the central nervous system (CNS). The translated wild-type huntingtin protein is a 350-kDa protein containing a polymorphic stretch of between 6 and 35 glutamine residues in its N-terminal domain [11]. For the length superior to 35 CAG repeat in the *htt* gene, the accumulation of polyglutamine in the protein leads to its aggregation in specific areas in the brain such as: striatum, cortex, thalamus, hypothalamus and the substantia nigra pars compacta. mHTT has a toxic gain of function that causes cell death via very different mechanisms, which still remain unclear. However, it is known to result in transcriptional dysregulation as well as mitochondrial dysfunction and energy deficits (for review see [1,12]). The accumulation of the mutant *htt* protein progressively compromises survival and normal neuronal functioning, primarily in the striatum (caudate/putamen). The mutant *htt* leads also to proteosomal dysfunction, induction of autophagy, release of Calcium from intracellular stores and excitotoxicity at extrasynaptic NMDA receptors. It particularly affects the GABAergic neurons, called medium spiny neurons (MSN) situated in the striatum, which have axonal projections to the globus pallidus and substantia nigra. They express Dopamine- and cAMP-Regulated neuronal PhosphoProtein of 32kDa (DARPP32). The progressive loss of these neurons is accompanied by a corresponding ventricular enlargement and gliosis

**(Figure 2)**. The disease progresses with the degeneration of cortical pyramidal neurons, mainly projecting to the caudate/putamen [13]. Many of the symptoms of HD result from the loss of inhibitory connections from the striatum to other structures such as the globus pallidus **(Figure 3)**. The cause of such specific regional and sub-population neuronal loss and the absence of cell loss in other tissues remain uncertain. Brain pathological hallmarks leading to 25% of brain weight loss in HD develop well before evident symptoms appear.



**Figure 2.** Brains' comparison by MRI between healthy and Huntington's disease subjects [14].

The most visible symptom of HD is the presence of involuntary jerky movements named chorea. During the first stage of the disease, the chorea dominates and when the disease progress, dystonia, rigidity and bradykinesia are also observed. Cognitive impairments also progressively appear as well as emotional disturbances marked in the most case by anxiety, memory loss, dementia, depression and psychosis [15]. They frequently lead to considerable distress and psychologic difficulty for patients, which have more prevalence than the general population to commit suicide. The unequivocal presence of chorea in a person with a family history or genetic confirmation of risk for HD forms the basis for clinical diagnosis. Recently, the American Academy of Neurology published guidelines to evaluate the motor and cognitive function, behavioral symptoms and functional capacity based on the Unified Huntington's Disease Rating Scale-Total Motor Score [16].



**Figure 3. Different areas involved in HD explaining symptoms.** The cortico-striatal system is essential for the execution of movements (left). In patients with HD, progressive degeneration of neurons in the striatum, and later also in cerebral cortex, disrupts function in the cortico-striato-pallidal circuit and induces severe impairments in both motor and cognitive functions. The striatal GABAergic projection neurons provide an inhibitory control of two major striatal output structures, globus pallidus and pars reticulata of the substantia nigra (not shown). Loss of these neurons, in animals with striatal lesions or in HD patients, results in disinhibition of pallidal outflow (right) [17].

There is no effective treatment for the progressive neurodegenerative process underlying HD, and management includes pharmacological symptomatic control of the movement disorder and psychiatric features, as well as non-pharmacological treatments, such as parenteral feeding and therapy services [18]. When the physiotherapy is not enough, tetrabenazine is the first choice of medication for uncomplicated chorea. Tetrabenazine is acting to decrease dopamine levels and can be helpful to reduce movement disorder but presents various side effects like the increase of depression or psychiatric disorders. Neuroleptics or benzodiazepines can also be prescribed and mood stabilizers such as anti-depressants and anti-anxiety reducing psychological dysfunctions are also proposed [18]. Unfortunately, many of these medications have adverse side effects that can worsen HD symptoms. In order to help to define the best treatment The Unified Huntington's Disease Rating Scale-Total Motor Score classified the level of evidence for drugs to reduce chorea based on a review of randomized clinical trials [19,20].

Nowadays, two approaches are under pre-clinical evaluation: disease modifying treatments, more particularly by reducing polyQ repeats and more recently by the suppression of the mRNA of the HTT gene with interference RNA (iRNA) [21]. Another approach consists in recent tissue engineering strategies to replace lost neurons by new ones obtained *in vitro* from stem cells. These methods need to be further improved and developed in order to be validated in the *in vivo* models of HD [18].

## **2. IN VIVO MODELS OF HUNTINGTON'S DISEASE**

Most animal models of HD fall into two broad categories, genetic and non-genetic. Historically, non-genetic models have dominated the field of HD research. Although George Huntington first described HD in 1872, researchers did not identify the actual genetic mutation responsible for the disease until 1993, which delayed the development of appropriate genetic models until the last decade [22].

The emergence of genetic and molecular technology allowed the development of animal models expressing a truncated [23] or full length [24,25] form of mutant htt (mhtt) (**Table 1**). Animal models are divided into two genetic categories: transgenic or knock-in. Transgenic models result from the random insertion of mutated human htt. The R6/1 and R6/2 transgenic mouse models were the first characterized [26] (**Table 1**). These mice express only mutant exon 1 of the human htt gene with different length repeats. These principal models containing only the truncated human mhtt gene are still the most used nowadays, together with yeast artificial chromosome's (YAC) models [24]. The latter consist on the cloning of an artificial yeast vector that contains the entire human mhtt with different expanded CAG repeats (YAC 46, YAC 72, YAC 128) which are then integrated into the rodent genome [27] (**Table 1**). Alternatively, the knock-in models result by the insertion in the htt mouse genome of the CAG repeats of human mhtt, which are then within the context of the rodent mhtt gene [28,29] (**Table 1**). In all of these animal models the pathophysiology of the disease appears in adult age as for the HD patients.

In any case none of these models exactly reproduce the human pathology. Moreover, the existence of various genetic animal models did not allow predicting HD symptoms. Indeed, we can believe that the progression is correlated with the number of CAG repeat length, but the mechanism of the disease seems to be more complicated. Moreover, it has been shown that therapeutic success in animal models is not always paralleled by clinical success in patients.

Animal models	Transgene product	CAG repeat length	Promoter and transgene expression	Method of cell death	Symptoms	Ref
<b>Truncated N-terminal fragment models</b>						
R6/1 mice	67 amino acids of N-terminal fragment (human HTT)	116	1kb human HTT promoter. Transgene expression ~ 31%	Aggregation and nuclear inclusion of htt	Slow progression of the symptoms, brain atrophy, dystonic movements, motor performance, grip strength and body weight loss	[30]
R6/2 mice	67 amino acids of N-terminal fragment (human HTT)	144	1kb human HTT promoter Transgene expression ~75%	Aggregation and nuclear inclusion of htt	High progression of the symptoms, brain atrophy, dystonic movements, motor performance, grip strength	[30]
<b>Full length HD models: knock-in models</b>						
Hdh <sup>Q92</sup> mice	Full length chimeric human HTT exon 1: mouse Htt	92	Endogenous mouse Htt promoter Transgene expression ~ 100%	Nuclear inclusion but not cytoplasm inclusion, aggregation of htt,	Not communicated	[31]
Hdh <sup>Q111</sup> mice	Full length chimeric human HTT exon 1: mouse	111	Endogenous mouse Htt promoter Transgene expression ~ 100%	Nuclear inclusion but not cytoplasmic inclusion, aggregation of htt	Not communicated but model to study juvenile HD	[31]
zQ175mice	Full length chimeric human HTT exon 1: mouse	188	Endogenous mouse Htt promoter Transgene expression ~ 100%	Nuclear inclusion aggregation of htt	Tremor, hypokinesia, abnormal gait, poor grooming, lost coordination, deficit in grip strength	[32]
<b>Full length HD models: transgenic models</b>						
YAC46 mice	Full length human HTT	48	Human HTT promoter and regulatory elements Tansgene expression ~40%	Increased intracellular calcium concentration	Any obvious abnormal behavior, electrophysiological abnormalities	[24]
YAC72 mice	Full length human HTT	72	Human HTT promoter and regulatory elements Tansgene expression ~40%	Very few nuclear inclusion, aggregation of htt	Symptoms YAC line dependent, tremor, ataxia, brain reduction	[24]
YAC128 mice	Full length human HTT	128	Human HTT promoter and regulatory elements Tansgene expression ~100%	Nuclear inclusion, aggregation of htt	Slow progression of the symptoms, brain atrophy, hypokinesia,	[33]
BACHD mice	Full length human HTT	97	Human HTT promoter and regulatory elements Tansgene expression ~100%	Progressive nuclear inclusion, aggregation of htt	Very slow progression of the symptoms, brain atrophy, dystonic movements, not body weight loss	[34]

**Table 1. Most used of genetic model of rodent animals to model HD available nowadays.**

Following early ideas that lesions of the striatum were responsible for HD, non genetic animal models used intrastriatal injections of neurotoxins

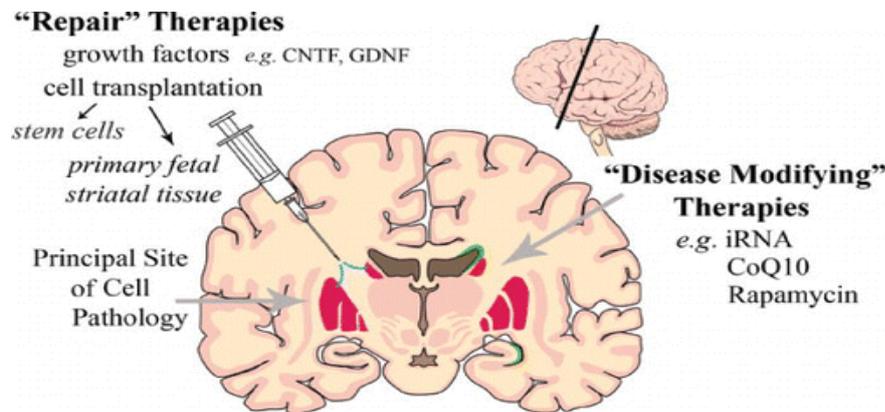
with glutamatergic targets such as kainic acid (KA) [35], ibotenic acid (IA), quinolinic acid (QA) [36] [37] and N-methyl-d-aspartate (NMDA) [38]. Indeed, glutamatergic excitotoxicity is involved in the pathophysiology of the disease. These lesions induced death of striatal medium spiny neurons similar to the neuropathology present in HD patients. The symptoms most commonly associated with the diseased state induced by the neurotoxins are loss of weight, possible tremor or seizures, eventual paralysis, recumbence, and often death representing the later stages of the disease. But these models do not allow investigation of disease progression or the mechanism of neuropathology because the htt protein does not present the mutation. In addition, genetic and non-genetic models need proper care and animals must be daily monitored. Immediately after toxin administration or in later stages in genetic models, euthanasia may be necessary for moribund animals. To reduce the number of animal experiments *ex vivo* models represents an excellent compromise between single cell cultures and animal studies.

### **3. EX VIVO MODELS OF HUNTINGTON'S DISEASE**

Another way for modelling HD consists in using organotypic brain slices, which have been also reported for modelling Parkinson's disease and cerebral ischemia. Brain slices are kept alive during several weeks in culture and represent a simple method to model the neurodegeneration and evaluate potential treatments before *in vivo* studies.

Brain slice models offer unique advantages over other *in vitro* platforms in that they can replicate many aspects of the *in vivo* context. Slices preserve largely the tissue architecture of the brain regions that they originated from and maintain neuronal activities with intact functional local synaptic circuitry [39]. Nevertheless, brain slices remain fragile, they can be easily distorted, and often flatten during the culture. In other word, organotypic brain slices are delicate and frequently become damaged during the preparative stages, so brain slice preparation and culture need experience. Functional outcome of a therapeutic strategy cannot be evaluated with this technique, but as brain slices can be maintained for a few weeks in culture they offer the possibility to screen a large quantity of therapeutic strategies. Pharmacologicals [40],

gene manipulation strategies with the goal to reduce the CAG repeat on HD [41] or injection of growth factors or cells can be evaluated for their neuroprotective or neurorestorative ability of damaged structures (**Figure 4**). Furthermore, organotypic slides represent a powerful tool for understanding the interaction between grafted cells and resident cellular matrix, or for comprehending the mechanisms of experimental treatments in HD [42].



**Figure 4.** The use of organotypic slices for the evaluation of innovative treatment in HD [43].

As *in vivo* models, organotypic brain slices of HD are divided in 2 categories: genetic and non-genetic models. Genetic models can be derived from adult animals already described above, presenting the MSN degeneration, or by transfecting the mutated *htt* within the organotypic culture. However, these organotypic cultures are difficult to generate due to the diminished neuronal plasticity and the fragility of these brains. Non-genetic models can be generated from pups or young animals by neurotoxin administration directly in the organotypic slice culture. The first HD organotypic model was developed in 1986 by injecting KA in the striatal organotypic slices [44]. Over the years, brain slice cultures have been successfully established with QA and/or 3-NPA, and KA after addition in the media of striatal organotypic slices to mimic the disease. In order to study later stage of onset disease all these neurotoxins have been injected in the frontal cortex, hippocampus or caudate nucleus for modeling HD in rat [45–47] (**Table 2**). But the use of neurotoxins leads to a heterogeneity in the results obtained, which must be taken in consideration. Furthermore, with that type of model, only the cellular aspect of HD can be studied, they cannot take

into account the genetic component of the pathology. Advantages are the speed and low-cost associated to this model when normal rodents are used [42].

Neurotoxins	Ex vivo models	Cells modification	Ref
Kainic acid (KA)	Striatal organotypic model	Enlargement of mitochondria, dilation of rough endoplasmic reticulum, presence of numerous vacuoles, glial fibrillary changes	[44]
Quinoleic acid (QA)	Corticostratial organotypic model	Excitotoxic damages, presence of numerous vacuoles	[48,49]
$\alpha$ -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate	Corticostratial organotypic model	Excitotoxic damages, presence of numerous vacuoles	[50]
3-nitropropionic acid (3-NPA)	Striatal and corticostratial organotypic model	No described but reductions of complex II–III Activity, mitochondrial function is impaired	[46]
QA + 3-NPA	Striatal organotypic model	No described.	[51,52]

**Table 2. Non genetic models for modeling Huntington with brain slices.**

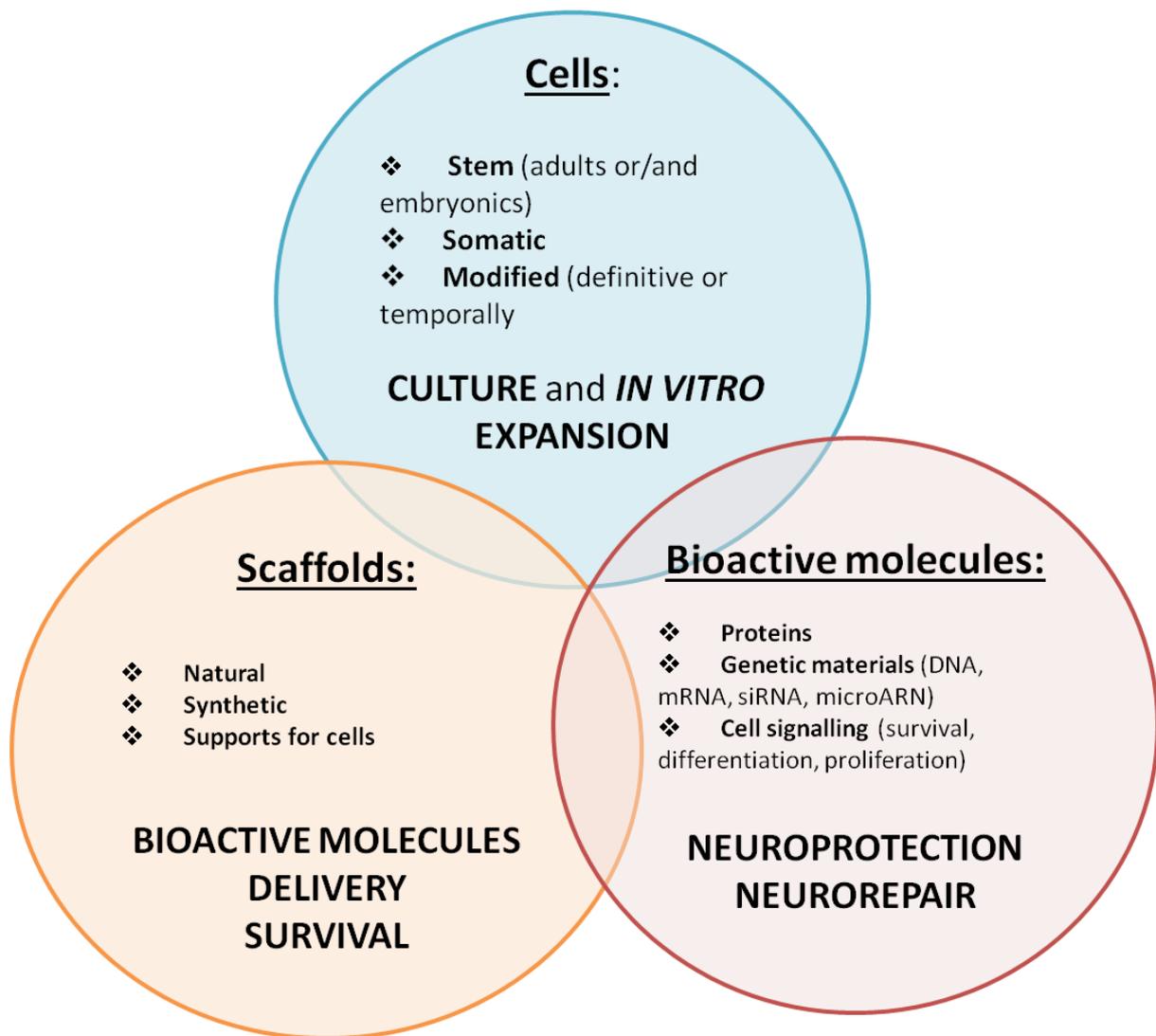
#### **4. EXPERIMENTAL TREATMENT IN HUNTINGTON’S DISEASE**

During the past years, many preclinical studies initially reported the efficacy of human fetal striatal tissue to replace and provide functional recovery in a variety of rodent and non-human primate models of HD. Some teams demonstrated the feasibility and the safety of this therapeutic strategy [53–57] and functional improvements were obtained in the study led by Bachoud-Lévi. They reported graft survival, which contained striatal projection neurons and interneurons, and received host-derived afferents [53,54]. But the survival is still very poor and the comparisons of these clinical trials are very difficult because of the heterogeneity in their design and the lack of controls. Nevertheless, the reported improvements in these trials appear to be modest and transient (for review see [58]). Moreover, the use of human fetal brain for striatal transplantation derived from elective abortions is limited by the lack of standardization inevitably correlated with the use of such a source as well as ethical, practical, and regulatory concerns and is dependent upon availability of donor tissue [59]. Stem cell-based

therapies can provide a limitless source of cells due to their self-renewal capacity and their neuronal differentiation potential, but good cell engraftment remains a drawback. On this basis, the development of cell replacement strategies for regenerative medicine and more particularly tissue engineering has been under light the last decade for HD.

#### 4.1 TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Tissue engineering and regenerative medicine are fields which have a unique tactic to solve clinical problems aforementioned by combining the principles of engineering, clinical medicine, biology and materials science [60]. Tissue engineering, according to the National Institute of Health (NIH), is a broad field which involves “ biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues”[61] and regenerative medicine as the development of “ therapies to restore lost, damaged, or aging cells and tissues in the human body”. Those approaches may include, but is not limited to, the use of soluble molecules [62], gene therapy, stem cells transplantation [63], reprogramming of cells. The strategic introduction of these bioactive and soluble molecules, as well as stem cells into the human body is directed not only to replacing tissue but also at inducing regeneration and revascularization by host tissue. Various cells can be considered in tissue engineering, stem or modified cells to replace lost neurons and somatic cells for their neuroprotective properties (**Figure 4**). Biomaterial scaffolding is often employed to provide a supporting spatial and biomolecular environment for transplanted cells. This approach named “top-down” in which cells are seeded onto a scaffold with biocompatible and biodegradable properties is the most used (**Figure 4**).



**Figure 5.** Schema of the three pillars of regenerative medicine. To bring tissue engineering into reality, it is crucial to sufficiently advance and combine the three. Currently, there is increasing recognition of the importance of cell culture methods, scaffolds supporting cells and drug delivery of signaling molecules.

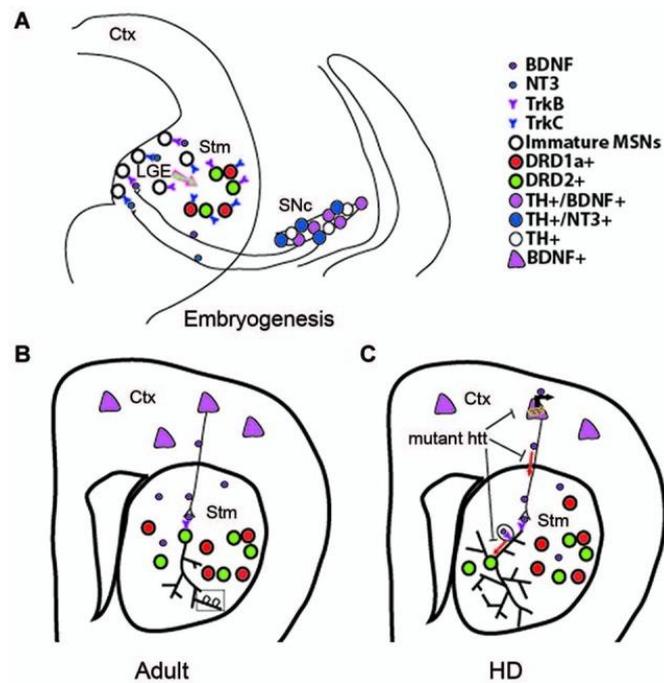
Regenerative medicine for brain stem cell therapy has been primarily developed with neuronal cell lines (PC12 cells), due to their availability and ease of expansion, or with cultured dorsal root ganglion (DRG) neurons and fetal NSCs, owing to their natural ability to integrate and differentiate within the brain.

The scaffold can also release bioactive molecules able to act as neuroprotectors. In the case of HD, two neurotrophic factors have been used for regenerative medicine: ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF). Clinical trials were performed using

CNTF-producing cells with stage 1 and 2 HD patients [64,65]. During this phase 1 study, subjects received one capsule implanted into the right lateral ventricle, and the capsule was exchanged every 6 months during 2 years. While the CNTF-induced sparing of striatal neurons and maintenance of intrinsic circuitry in animal models was impressive, the effect in human was less than that seen in rodents. Finally, human clinical trials did not present relevant positive effects, and progressively the supplementation of CNFT in human has been given up. To our knowledge, no clinical trial has been conducted with BDNF.

#### 4.2 POTENTIAL THERAPEUTIC USE OF THE NEUROTROPHIC FACTOR BDNF

Neurotrophic factors are essential for the survival of the central nervous system neurons and demonstration of their reduced availability in HD indicates that they may play an important role in this disorder. Indeed, the reduction of BDNF in HD contributes to the disease onset and or progression [66,67]. BDNF is essential in sustaining the physiological processes of normal intact adult brain [68] and more particularly for GABAergic striatal neurons (**Figure 5**). Indeed, although widely expressed in the adult mammalian central nervous system, BDNF is particularly abundant in the hippocampus and cerebral cortex where it is anterogradely transported to its striatal targets via the corticostriatal afferents [69]. Several evidences demonstrate the role of BDNF in the maturation of striatal neurons and how BDNF promotes the survival of DARPP-32 positive neurons [70,71].



**Figure 6. Role of BDNF in developing, adult, and HD striatum.** (A) A proposed model showing that BDNF and NT3 anterogradely transported from mesencephalic dopaminergic neurons regulate survival of immature neurons in the indirect and direct pathways, respectively. Ctx, cerebral cortex; Stm, striatum; SN, substantia nigra. (B) Cortical BDNF in the adult striatum mediates dendritic complexity and spine number and morphology. (C) Mutant htt reduces BDNF-TrkB signaling by inhibiting BDNF gene transcription, axonal transport of vesicles containing BDNF, retrograde dendritic transport of TrkB-positive endosomes to the cell body [72].

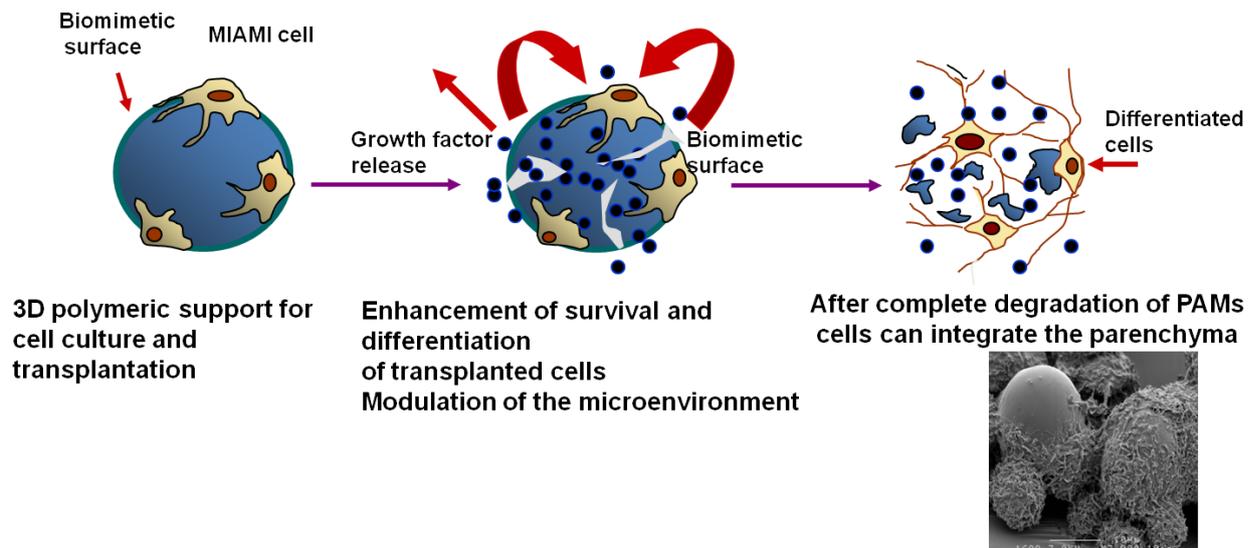
The supplementation of BDNF in the case of HD has been evaluated and promising results show that BDNF treatment prevent striatal degeneration in a chronic model of HD.[73]. More interestingly, the supplementation of BDNF protein increases the survival of enkephalin-immunoreactive striatal neurons, reduces striatal interneuronal loss and improves motor function in HD animal models [74–76]. Despite these data, the BDNF supplementation raises a number of problems such as the stability of BDNF as it cannot cross the blood-brain-barrier. Moreover, an uncontrolled BDNF administration may interfere with neuronal plasticity and even give rise to serious side effects [77]. One way to avoid this limitation is the encapsulation of BDNF within the scaffold necessary for tissue engineering.

### 4.3 POTENTIAL THERAPEUTIC USE OF SCAFFOLDS

To overcome the poor cell survival and engraftment usually observed after transplantation, several strategies have been developed and among them,

two methods seem particularly promising: *in situ* controlled drug delivery and implantation of cells adhered on biomaterial-based scaffolds. Such scaffolds should provide an adequate 3D support for transplanted cells, thereby increasing cell survival and even guiding cell differentiation and fate *in vivo* [78,79]. However, delivery of cells with scaffolds to the damaged brain still remains challenging due to practical limitations of delivery [80]. Ideal properties of a scaffold for brain tissue engineering are biocompatibility, very small size, controlled biodegradability with non-toxic derivative products, and three-dimensional (3D) matrices with appropriate mechanical properties to mimic the extracellular matrix [81,82].

An innovative scaffold for tissue engineering combining these ideal properties with a biomimetic 3D approach and the release of bioactive molecules has been developed in our laboratory. This scaffold or microcarriers named Pharmacologically active microcarriers (PAMs) are constituted of a synthetic polymer based on poly(lactic-co-glycolic acid) (PLGA) [78]. They are obtained by a non-denaturing solid-in-oil-in-water (s/o/w) emulsion evaporation/extraction technique. The protein is first nanoprecipitated with poloxamer 188 a biocompatible hydrophilic polymer that protects the protein against irreversible denaturation [83]. PAMs are biodegradable and biocompatible with a mean size of 60  $\mu\text{m}$ , covered by a biomimetic surface providing a 3D support for the cells and delivering a therapeutic protein in a prolonged manner. All these combined properties stimulate the survival and differentiation of the transported cells [84]. Their small size allows their implantation into the brain through a needle or catheter and after the complete degradation of the polymer the cells may integrate the parenchyma. These PAMs have been successfully employed for different tissue engineering strategies, in neurodegenerative disorders, cerebral ischemia, myocardial infarction, and cartilage repair (**Figure 6**) [85,78,86,83,87]. For these strategies the appropriate growth factor and extracellular matrix protein have been combined to either progenitors or stem cells. In this work we will use stem cells combined with PAMs presenting a biomimetic surface of laminin, which stimulates neuronal differentiation [88] and delivering BDNF as a therapeutic strategy for HD.



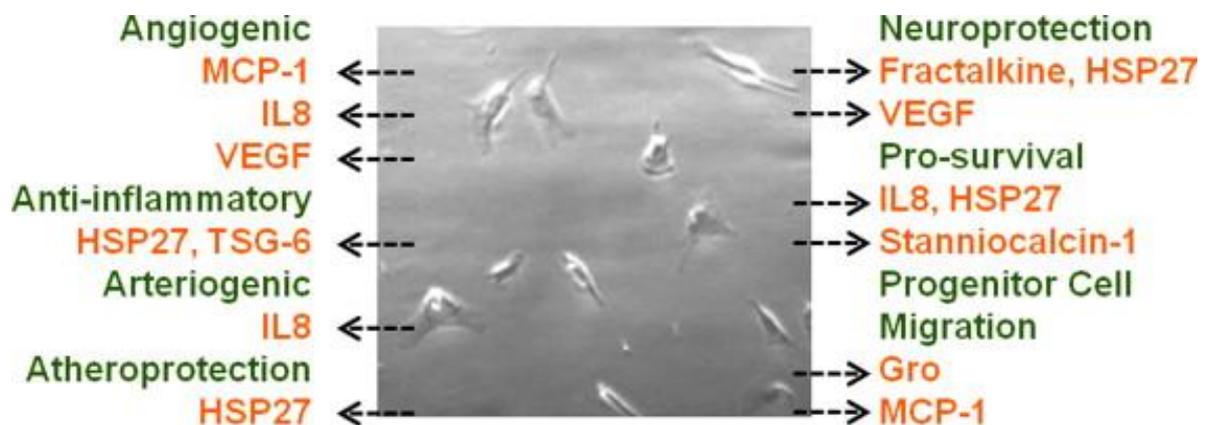
**Figure 7.** Concept of PAMs: the biomimetic surface of PAMs is obtained by coating their surface with extracellular matrix proteins that can favor cell adhesion. During their formulation, the encapsulation of neurotrophic factor is performed.

#### 4.4 . POTENTIAL THERAPEUTIC USE OF STEM CELLS

During the last decade, several preclinical experiments have used cell replacement strategies in order to restore MSN using HD animal models. Different human stem cell sources are being actively explored for potential cell replacement therapy including embryonic stem cells (ESCs), induced pluripotent or neural stem cells (iPSC or NSC), fetal and adult neural precursors and mesenchymal stem cells (MSCs). Both ESC, iPSC have been successfully committed into MSN *in vitro* and then grafted into rodent models of HD [37,89,90]. But after human ESC transplantation into rat brains, tumor formation has been reported, which was not the case for iPSC-MSN-derived cells, which were further committed within this lineage. In addition, the ethical issues related to the use of ESCs and the lack of availability of fetal neural precursors drive us to focus in other cell sources. To find the best way to obtain the most important benefits with less ethical and practical constraints, mesenchymal stem cells have been investigated.

Human MSCs, as Friedenstein reported, are capable of differentiating into cells deriving from the mesodermal layer, such as osteoblasts, chondrocytes and adipocytes. Along with their self-renewal property, MSCs secrete tissue repair factors, such as growth factors, which affect the

surrounding microenvironment to promote angiogenesis, decrease inflammation, and enhance tissue repair [91]. In this way, MSCs are being widely evaluated in many clinical trials for cell therapy showing the feasibility of this approach. Recent advances in stem cell biology hold great promise in the development of MSCs-based therapy for tissue engineering. It was also demonstrated that these cells could differentiate to an ectodermal neural/neuronal phenotype, particularly under the influence of specific factors [85] [79,92] enabling their use for cell therapy for neurodegenerative disease including HD. The principal limitation is that MSCs are a heterogeneous population with cells presenting different differentiation properties. To avoid these limitations a homogenous subpopulation of MSCs, named “Marrow-Isolated Adult Multilineage Inducible” (MIAMI), which present an unique genetic profile expressing several pluripotency markers (Oct4, Sox2, Nanog, SSEA4) and secrete many varied cytokines (**Figure 7**) are interesting for the treatment of neurodegenerative disorders [93,94].



**Figure 8.** Soluble factors secreted by MIAMI cells involved in the tissue repair. [93,94]

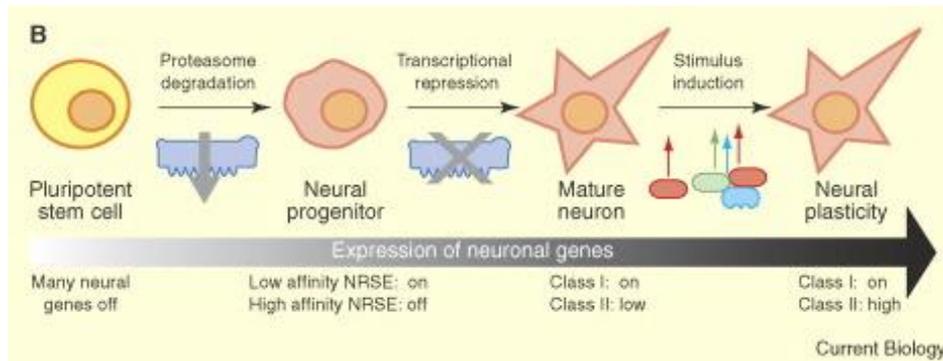
They are able to generate cells derived from all three embryonic germ layers and cultured on fibronectin; they are capable of differentiating into neuron-like cells under treatment with various factors. After treatment, the cells show neurites, express neuronal factors and present some electrophysiological characteristics similar to those observed in mature neurons [95]. Recently, in a rat model of Parkinson's disease (PD), striatal implantation of MIAMI cells pre-committed towards the dopaminergic phenotype adhered to microsphere releasing bioactive molecules improved

stem cell survival and showed dopaminergic differentiation. This led to the protection/repair of the nigro-striatal pathway and to functional recovery of the PD rats. Furthermore, implantation of pre-treated MIAMI cells also induced functional recovery in PD rats, probably due to the release of glial cell line derived neurotrophic factor (GDNF) [88]. But MIAMI cells' ability to differentiate into neurons, although better than the simple MSC needs to be improved.

#### 4.5 POTENTIAL THERAPEUTIC OF SMALL INTERFERING RNAs FOR MSC DIFFERENTIATION

The conventional methods of generating neurons from MSCs, through bolus supplementation of small molecules or neurotrophic factors (growth factors: GF), still lack in efficiency in neural conversion and lineage selection. One possible reason may be the inadequacy of GFs to control gene expression. Cell differentiation may be achieved by RNA interference (RNAi) strategies and more particularly by small interfering RNA (siRNA), which selectively knock-down the expression of only a few pivotal genes. Indeed, siRNAs are synthetic duplex of 21–23 nucleotides, approximately 7.5 nm long and 2 nm in diameter, which are capable of specifically targeting one gene and silencing it in a post-transcriptional way. SiRNA are rapidly taken up into an enzyme complex, RNA induced silencing complex (RISC), that degrades the mRNA through guidance to a specific target mRNA resulting in specific gene silencing. At RISC one siRNA strand is taken into the effector complex, the catalytic subunit Argonaute2, and then serves as a template, guiding the hydrolysis of complementary or near complementary mRNA sequences [96]. Initially siRNA emerged as a potential therapeutic treatment for cancer. Although current applications in stem cells remain largely restricted to studies on molecular pathways and signalling, RNAi can be used as a biomedical strategy to direct lineage-specific differentiation of stem cells for therapeutic purposes [97]. One key factor that can possibly be adapted into the siRNA strategy for directing neuronal differentiation of neural stem cells, is the repressor element 1 (RE-1) silencing transcription factor (REST) [98]. In most differentiated non-neuronal cells and uncommitted neural stem cells, REST functions as a transcriptional repressor for a myriad of neuronal

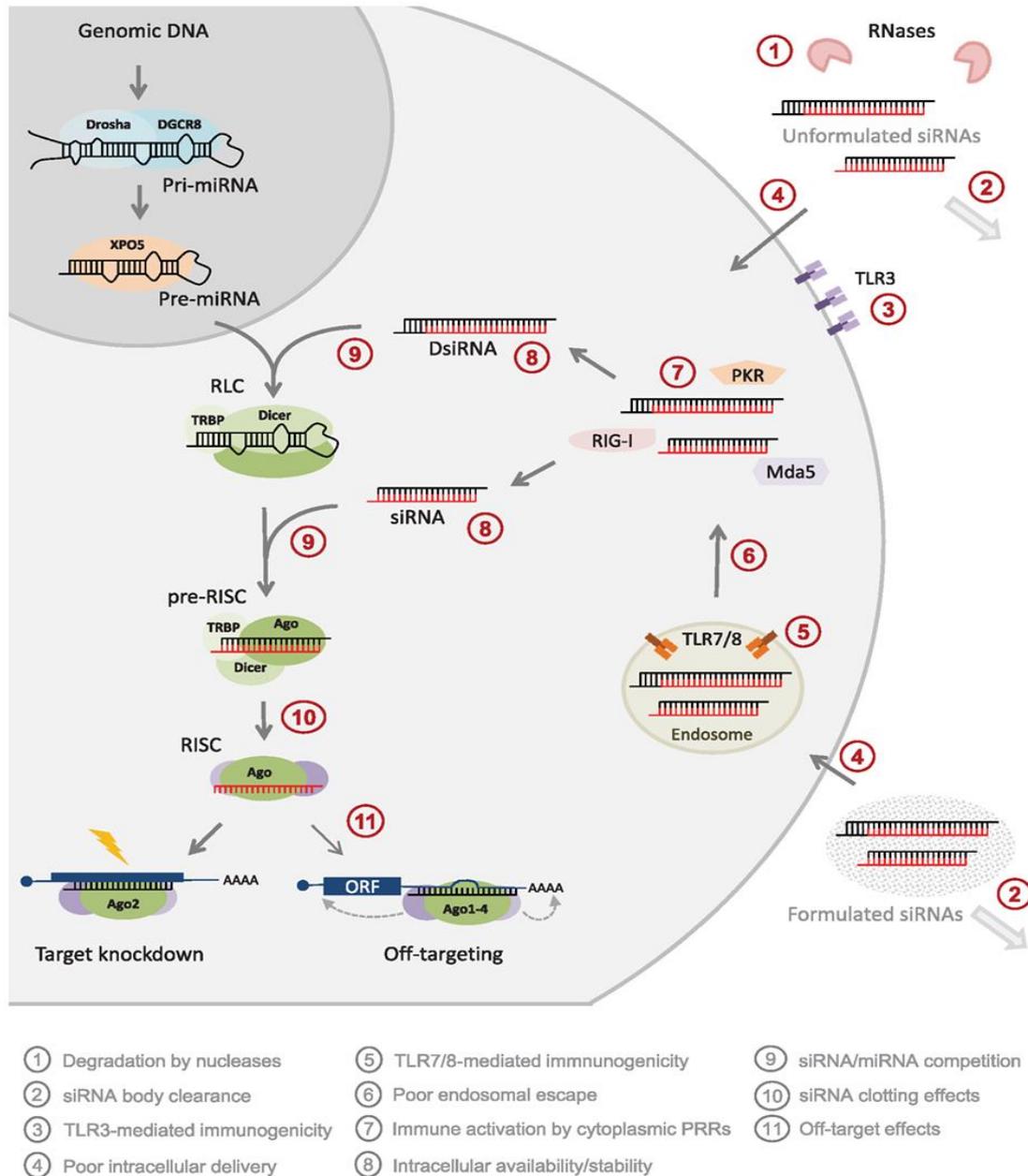
specific genes such as ion channels, synaptic vesicles proteins, and neurotransmitter receptors by binding to a highly conserved DNA sequence known as RE-1. During neurogenesis, REST is rapidly down regulated in embryonic stem cells (ESCs) and neural stem cells upon differentiation into neurons [99] (**Figure 8**).



**Figure 9.** REST expression during the neurogenesis [100].

Conversely, induced down regulation of REST has been shown to promote neuronal commitment in mouse ESC and mouse MSCs. Specifically, the knockdown of REST in ESCs induced neural progenitors formation [101] (**Figure 8**) and when applied to MSCs, cross-lineage differentiation to neurons was observed [102,103]. Although the knockdown of REST holds great potential, its therapeutic applications in neuronal differentiation is hindered by poor cellular uptake of siRNA molecules and their rapid enzymatic degradation [104]. siRNAs' molecular weight (~13 kDa) and strong anionic charge due to the presence of aphospho-diester backbone (~40 negative phosphate charges), make them incapable of freely crossing the cell membrane. The electrostatic repulsion from the anionic cell membrane surface results in the failure of siRNA to passively diffuse through the cell membrane (**Figure 9**). Moreover, the synthetic siRNA molecules show low stability in physiological fluids, poor tissue/cell specificity, and rapid clearance [105]. Therefore, successful siRNA therapeutics requires effective and safe carrier systems to overcome the inherent limitations of siRNA and achieve maximum gene silencing effect. In the last decade, two different approaches for siRNA delivery have been developed: viral and non-viral vectors. In particular, the advantages of non-viral vectors are their low immunogenicity, their relatively low production cost and reproducibility

potentially. These reasons make them promising carriers for siRNA delivery [106].



**Figure 10.** The benefits and limitations of synthetic siRNA application. The representation of the limitations involved in the siRNA delivery. [107]

## 5. NANOCARRIERS

### 5.1 DIFFERENT TYPES OF NANOCARRIERS

Nanocarriers (NCs), including nanoparticles and nanocapsules were first developed for the potential delivery of therapeutic factors such as

chemotherapeutic agents to tumors or, when combined to stem cells, mostly for stem cell imaging [108]. First, the use of nanocarriers aims to protect an active ingredient against a potential degradation, and secondly to modify the natural distribution of the active substance in the body and in cells. It is theoretically possible to accumulate the active ingredient to the desired site of action and away from undesirable sites to limit side effects. NCs ranging from 1 to 1000 nanometer sizes are divided into 2 main categories:

- Organic NCs which include liposomes, lipid nanoparticles, solid nanoparticles and dendrimers.
- Inorganic NCs with quantum dots, carbon nanotubes, iron or gold nanoparticles [109].

The organic NCs and more particularly lipid based NCs are interesting to transfect cells because lipid based nanoparticles can contain lipids present in the biological membranes which help the entry of nanoparticles. Cationic charges contained in some lipids are able to interact with nucleic acid. In addition, the risk of undesirable immunogenic reactions to lipids is also relatively lower than most of the polymeric materials which generally have higher molecular weights [110]. Furthermore, some clinical trials have been conducted with siRNA and lipids based nanoparticles [111,112]. To our knowledge, clinical trials with nanoparticles and MSCs have not been performed in HD. When compared with liposomes, lipid-based nanoparticles such as solid lipid nanoparticles generally have solid, lipophilic core regions so it is inherently difficult to truly encapsulate the hydrophilic, poly-anionic RNA molecules. As a result, there are relatively few lipid nanoparticles for RNA delivery [113,114].

## 5.2 LIPID NANOCAPSULES

Lipid nanocapsules (LNC) are nanocarriers developed and recently patented. These nanocapsules are constituted by oily core of tryglicerides and a shell made of surfactants particularly polyethylene glycol hydroxystearate. They are obtained by a phase inversion temperature dependent process. This

solvent-free process requires little energy and allows easy large-scale transposition [115].

The formulation is based on a simple process, named emulsion's phase inversion, developed and patented in 2002. This is realized by oil in water emulsion (O / W) using the various constituents described above. This emulsion is subjected to an increase of temperature which induces a change in the hydrophilic / lipophilic balance. Several temperature cycles (between 50 and 90°C) are produced and the addition of cool water final stabilizes and solidifies LNCs.

Previous studies demonstrated the possibility to encapsulate plasmid DNA within the LNCs to develop a gene therapy strategy [116]. For this, the DNA is complexed with cationic lipids by electrostatic interactions leading to formation of complexes called lipoplexes which are added to other components of the LNC. Moreover, the phase inversion temperature was reduced to avoid degradation of the plasmid [116]. This strategy has demonstrated the capacity of LNCs to transfect in the *in vivo* models of glioblastoma [117–119].

### 5.3 SOLID SPAN NANOPARTICLES

Solid span nanoparticles have been recently developed and patented [120]. These nanocarriers are based on sorbitan esters, which are components widely used in the pharmaceutical industry due to its non-ionic surfactant properties at low concentrations. These nanoparticles can be prepared using a simple, one-step and easily scalable procedure (<https://www.youtube.com/watch?v=fda9CtJ5zF0>) and they can associate different components and/or bioactive molecules. The internal structure of this nanocarrier is not an aqueous inner space surrounded by a lipid bilayer nor it is based on nanoemulsions, but rather it is a homogenous nanoparticulate solid structure. It is also possible to incorporate various additional components. These additional components allow to modulate the nanosystem features conferring a great versatility in terms of physical-chemical characteristics and interaction with other components, and facilitate

the incorporation of active ingredients: hydrophilic and lipophilic nature [121].

Solid span nanoparticles (SP) based on sorbitan monooleate (Span® 80) have been specifically adapted to provide effective DNA association [122]. They have the capacity to provide higher *in vivo* transfection levels than adeno-associated viral vectors (AAV2). Indeed, it has been recently patented their use in gene transfer or gene delivery and described an *in vivo* proof of concept in a disease animal model showing the safety and clinical efficacy of a gene therapy approach based on these nanocarriers [123].

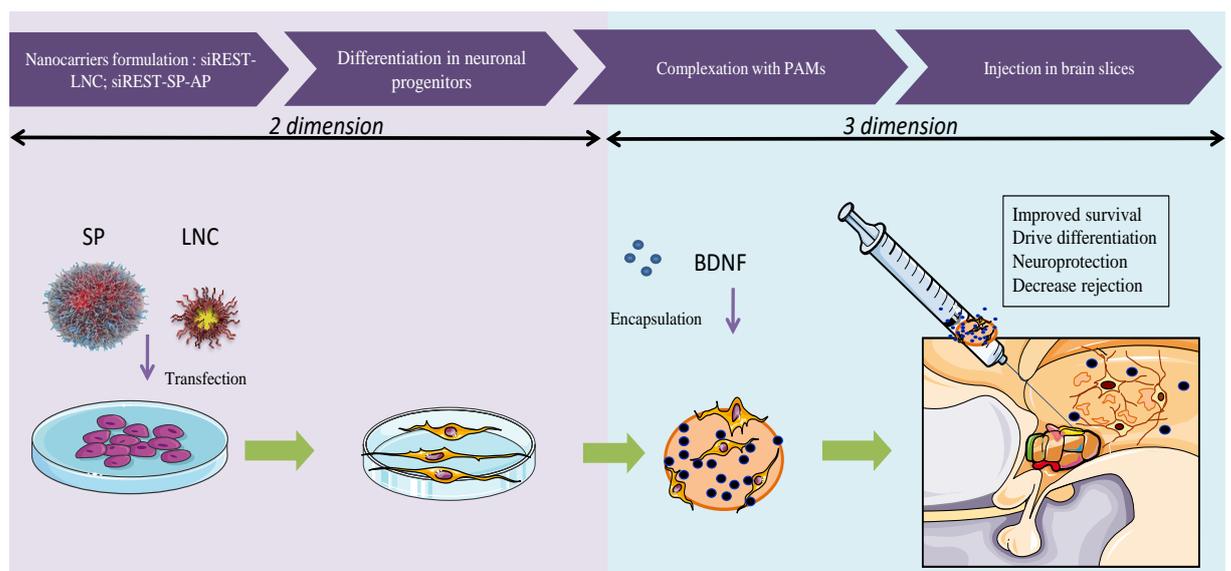
## **6. OBJECTIVES OF THIS THESIS**

The evaluation of a regenerative medicine strategy may be easily performed in a simple *ex vivo* model before pre-clinical study *in vivo*. Although, the transplantation of MSCs showed promising results the differentiation state needs to be improved. In order to ameliorate this point, the homogenous more pluripotent population of MIAMI cells seem to be a good option. Although MIAMI cells can be committed into neural/neuronal precursors their maturation still needs to be safely improved. Consequently, the transfection of siREST with nanoparticles seems to be a safe way to help induce a neuronal differentiation process. However, in order to control the behaviour of the cells after transplantation and enhance/maintain their differentiation their combination to PAMs delivering BDNF seems appropriate. Furthermore, the delivered BDNF may also induce the neuroprotection of the damaged tissue. In consequence, this study aims to develop an innovative and safe regenerative medicine strategy combining siREST nanoparticle-engineered MSCs, combined to PAMs with a laminin biomimetic surface and delivering BDNF for HD **(Figure 10)**.

The principal objectives of this study are:

- The development of a simple *ex vivo* HD model to evaluate a tissue engineering strategy.

- The development, the understanding and the optimisation of two nanocarriers (LNC and SP) transporting a siRNA (siREST).
- The evaluation of their efficacy to deliver this siRNA into MSCs and the selection of the best nanocarrier for this application.
- The evaluation of the capacity of si-REST to induce a neuronal commitment and its capacity to improve the differentiation of MIAMI cells
- The evaluation of the neuroprotective effect of modified MIAMI cells combined with PAMs coated with laminin and delivering BDNF
- The evaluation of the regenerative capacity of these complexes (MIAMI/LM-PAM-BDNF) and their engraftment in the *ex vivo* HD model



**Figure 11.** The tissue engineering strategy envisaged in this work.

This manuscript reports a bibliographic work with a review (submitted for publication) and results obtained during this study organized into 3 chapters.

The first chapter is entitled "MODELIZATION OF HUNTINGTON DISEASE". The goal was to develop an innovative and simple model of HD

without the addition of neurotoxins into the media and to model the neurodegeneration of medium spiny neurons (Publication n°1).

Then the second chapter "INNOVATIVE STRATEGY TO MODIFY STEM CELLS FOR TISSUE ENGINEERING" shows by the development, the characterization and the optimisation of two nanocarriers capable of transfecting mesenchymal stem cells and the evaluation of the effect of the siRNA against REST (named siREST) (Publication n°2).

Finally, the third chapter named "PHARMACOLOGICALLY ACTIVE MICROCARRIERS AS INNOVATIVE STRATEGY FOR COMMITTED MIAMI CELLS" describes different protocols used for the differentiation and the interaction between committed MIAMI with siREST and PAMs and a preliminary evaluation of their engraftment (Publication n°3).

A general discussion comparing the existing strategies will close these studies and open new prospects.

## REVIEW

With the aim to propose an innovative cell-based regenerative medicine strategy for the neurodegenerative disorder HD, stem cells or neuronal progenitors derived from these cells can be considered. The progress in cell engineering by reprogramming/programming cells to obtain induced pluripotent stem cells or induced neuron cells have revolutionized this field. It is however crucial to better monitor their proliferation, improve their survival and differentiation and hence ameliorate their engraftment after transplantation.

To direct stem cell fate, a delicate control of gene expression through RNA interference (RNAi) is emerging as a safe epigenetic approach. RNAi allows selecting specific knock-down the expression of mRNAs by degrading them. This epigenetic modification is quite simple, does not need genetic manipulation, is transitory and is now quite well understood. Nonetheless, nucleic acids need to be vectorized to be protected and to be able to cross biological membranes. Thus, the RNAi used for gene suppression strategies in many cell models are conventionally mixed with cationic lipids. Their toxicity limits their use and thus many nanocarriers have been designed to carry RNA inside cells.

A bibliographic research work has been undertaken here to identify new tissue engineering strategies currently under evaluation for HD. The first part of this study is focused on the possible source of cell for tissue engineering, presenting their advantages and disadvantages. A detailed review of the different formulations available for RNAi transport within stem cells, their mode of action and some examples of their use to control cell behavior follow. In the last part, innovative tissue engineering strategies using stem cells, biomaterials and epigenetic cell regulation are reported and discussed.

*This work is submitted for publication in Biomaterials*

*Revisions demanded*

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# **NANO AND MICROCARRIERS FOR STEM CELL THERAPY OF NEURODEGENERATIVE DISORDERS: APPLICATION TO HUNTINGTON’S DISEASE**

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## **ABSTRACT:**

The potential treatments for neurodegenerative disorders will be revolutionized by the transplantation of stem cells or neuronal progenitors derived from these cells. It is however crucial to better monitor their proliferation, improve their survival and differentiation and hence ameliorate their engraftment after transplantation. To direct stem cell fate, a delicate control of gene expression through RNA interference (RNAi) is emerging as a safe epigenetic approach. The development of novel biomaterials (nano and microcarriers) capable of delivering proteins, nucleic acids and cells, open the possibility to regulate cell fate while achieving neuroprotection and neurorepair. This review first provides an overview of stem cell therapy for the neurodegenerative disorder Huntington's disease. Within that context, an integrative discussion follows of the control of stem cell behaviour by RNAi delivered by different nanocarriers in vitro prior to their transplantation. Finally, combined in vivo strategies using stem cells, biomaterials and epigenetic cell regulation are reported.

## **KEYWORDS**

Stem cells; nanoparticles; microcarriers; tissue engineering; RNAi

## **ABBREVIATIONS**

HD, Huntington's disease; MSN, medium spiny neurons; ESCs, embryonic stem cells; iPS, induced pluripotent stem cells; NSC, neural stem cells; MSC, mesenchymal stem cells; RNAi, interference RNA; siRNA, short interfering RNA; miRNA, micro-RNA; NPs, nanoparticles; REST, repressor element-1 silencing transcription factor; PLGA, polylactide-*co*-glycolide; BDNF, brain-derived neurotrophic factor; ECM, extracellular matrix.

## 1. INTRODUCTION

### 1.1 HUNTINGTON'S DISEASE

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder with a general prevalence of about 10 per 100,000 births [1–3]. HD appears in middle life leading to death 15-20 years later and involves the triad signs and symptoms: involuntary movement disorders called Huntington's chorea, cognitive impairment and psychiatric manifestations. This disorder is characterized by an unstable repetition of triplet cytosine-adenine-guanine (CAG) of the Huntingtin gene, translated at the protein level by the polyglutamine expansion at the NH<sub>2</sub>-terminal part of the protein huntingtin (HTT)[4]. The gene is considered as normal when it contains less than 27 CAG repeats and generally more than 40 repeats defines the adult-onset HD, with people developing the disease at 30-40 years of age. However, people with 36-39 CAG repeats are at a risk of developing all the HD symptoms [5]. Conversely, a larger number of repeats is usually associated with an earlier onset of signs and symptoms [6]. Aggregation of the mutated htt results in transcriptional dysregulation as well as mitochondrial dysfunction and energy deficits (for review see [1,7]). The accumulation of the mutant htt protein is excitotoxic, therefore it progressively compromises survival and normal neuronal functioning, primarily in the striatum (caudate/putamen). It particularly affects the GABAergic neurons, called medium spiny neurons (MSN), which have axonal projections to the globus pallidus and substantia nigra. They express Dopamine- and cAMP-Regulated neuronal PhosphoProtein of 32kDa (DARPP32). The progressive loss of these neurons is accompanied by a corresponding ventricular enlargement and gliosis. The disease progresses with the degeneration of cortical pyramidal neurons, mainly projecting to the caudate/putamen [8].

Currently, no treatment can prevent the disease or stop the progression of HD. Recently, the American Academy of Neurology published guidelines for the pharmacological symptomatic treatment of HD [9]. It classifies the level of

evidence for drugs to reduce chorea based on a review of randomized clinical trials using the Unified Huntington's Disease Rating Scale-Total Motor Score (UHDRS-TMS) to choose the best treatment. Tetrabenazine, acting to decrease dopamine levels, is the most prescribed treatment but in some cases, antipsychotics can help to reduce chorea. Anti-depressants and anxiolytics can be prescribed to reduce psychological dysfunctions. Unfortunately, many of these medications have adverse side effects that can worsen HD symptoms.

During the past years, many preclinical studies initially reported the efficacy of human fetal striatal tissue to provide functional recovery in a variety of rodent and non-human primate models of striatal neuronal loss. On this basis, some clinical trials then assessed the potential of fetal neural transplants for the treatment of HD. In this review, we will briefly outline the emergence of fetal neural therapy replacement and its limitations. We will continue by describing the pre-clinical studies performed with different stem cells, which represent an alternative cell source, and we will comment on their limitations, the most important one being their limited engraftment. We will further provide an integrative description and discussion of nanoparticles transporting interference RNA therapeutic (RNAi) to initiate cell differentiation and increase survival in order to avoid some of the limitations described above. Finally, in order to improve their engraftment within the brain parenchyma, increase neuroprotection and neuro-repair, we will present combining approaches with cell modified with RNAi therapeutics nanoparticles and drug delivery devices.

## 1.2 CELL TRANSPLANTATION

To replace the degenerating neurons in HD patients, some teams explored the transplantation of fetal human brain tissue. They demonstrated the feasibility and the safety of this technique [10–14]. Functional improvements were obtained in the study led by Bachoud-Lévi. They reported graft survival, which contained striatal projection neurons and interneurons, and received host-derived afferents [10,11]. Comparisons of these clinical trials are very difficult because of the heterogeneity in their design, lack of controls, unblinded nature, and different methods used to assess clinical and motor outcome in each. Nevertheless, the reported improvements in these trials appear to be modest and transient (for review see [15]). Indeed, limitation of cell therapies resulted from

the extent of damage affecting HD patients. Moreover, the use of human fetal brain for striatal transplantation derived from elective abortions is limited by ethical, practical, and regulatory concerns and is dependent upon availability of donor tissue [16]. Besides the limited supply of human fresh fetal tissue, the strategy of striatal transplantation is further complicated by the lack of standardization inevitably correlated with the use of such a source.

One of the challenges is to identify an alternative cell source able to differentiate into MSN such as pluripotent stem cells (PSC), which are currently under investigation, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC). Adult neural stem cells (NSCs) and mesenchymal stem cells (MSCs) are also alternative candidates for regenerative medicine **(Figure 1)**.

### 1.3 PLURIPOTENT STEM CELLS FOR CELLULAR THERAPY

PSC are defined by their capacity of self-renewal thus offering the possibility of an unlimited supply of cells and by their pluripotency that is their ability to differentiate into all three germ layers. *In vitro* differentiation of pluripotent stem cells into GABAergic MSNs generally follows a multi-step process: i) induction of neural lineages; ii) regional patterning and the differentiation of neural progenitor cells; and iii) specialization of a mature neuronal subtype. GABAergic MSNs can be generated using several culture methods, including co-culture on feeder cells and suspension culture as embryoid bodies. Additionally, *in vitro* differentiation of pluripotent stem cells into MSNs requires cytokine signalling, controlled timing, duration, and concentration of exposure to developmental factors and reliable markers to identify mature MSNs capable to acquire electrophysiology properties.

#### 1.3.1 Embryonic stem cells

Human ESCs (hESCs) derive from the inner cell mass of blastocyst stage embryos created by *in vitro* fertilization. Aubry et al., were the first ones to describe the differentiation of hESC into MSNs [17]. The authors proposed a novel 3 step-protocol to obtain striatal progenitors using 2 major proteins involved in the striatum ontogenesis: Sonic Hedgehog (SHH) and Dickkopf-

related protein 1 (DKK1). The caudate/putamen or striatum derives principally from the lateral ganglionic eminence (LGE) [18], with DLX2-positive cells differentiating into MSNs, the most important population in the striatum. The injection into an adult rat model of HD (quinolinate-lesioned adult rats) of an early striatal differentiation stage of these hESCs presented massively over-proliferating neural progenitor cells. However, later stage striatal progenitors engrafted into the quinolinate-lesioned striatum and proved the feasibility of this cellular therapy [17]. The optimal time and dosage of SHH pathways in these cells was further determined as well as a major improvement to obtain the optimal commitment stage of the cells for transplantation [19]. When these LGE-like ESC-derived cells were induced to terminal GABAergic differentiation *in vitro*, the majority of cells expressed GABA (90%) and DARPP32 (81%) and presented appropriate neuronal characteristics as determined by HPLC and whole-cell patch-clamp. In addition, there was no sign of massive overgrowth or tumor formation up to 16 weeks after transplantation. The graft-derived GABAergic projection neurons were integrated into the host neural circuitry, receiving dopaminergic inputs from the midbrain and glutamatergic inputs from the cortex while projecting fibres to the substantia nigra [20]. However, ESC research is laden with ethical concerns, particularly regarding the ideas of personhood and justice toward humankind that arise from dealing with human life in one of its earliest forms, the embryo. It is a controversial international issue, and many governing bodies have either banned the research altogether or placed restrictions on what may be done with embryos and ESCs, limiting their use for cell therapy.

### 1.3.2 [Induced pluripotent stem cells](#)

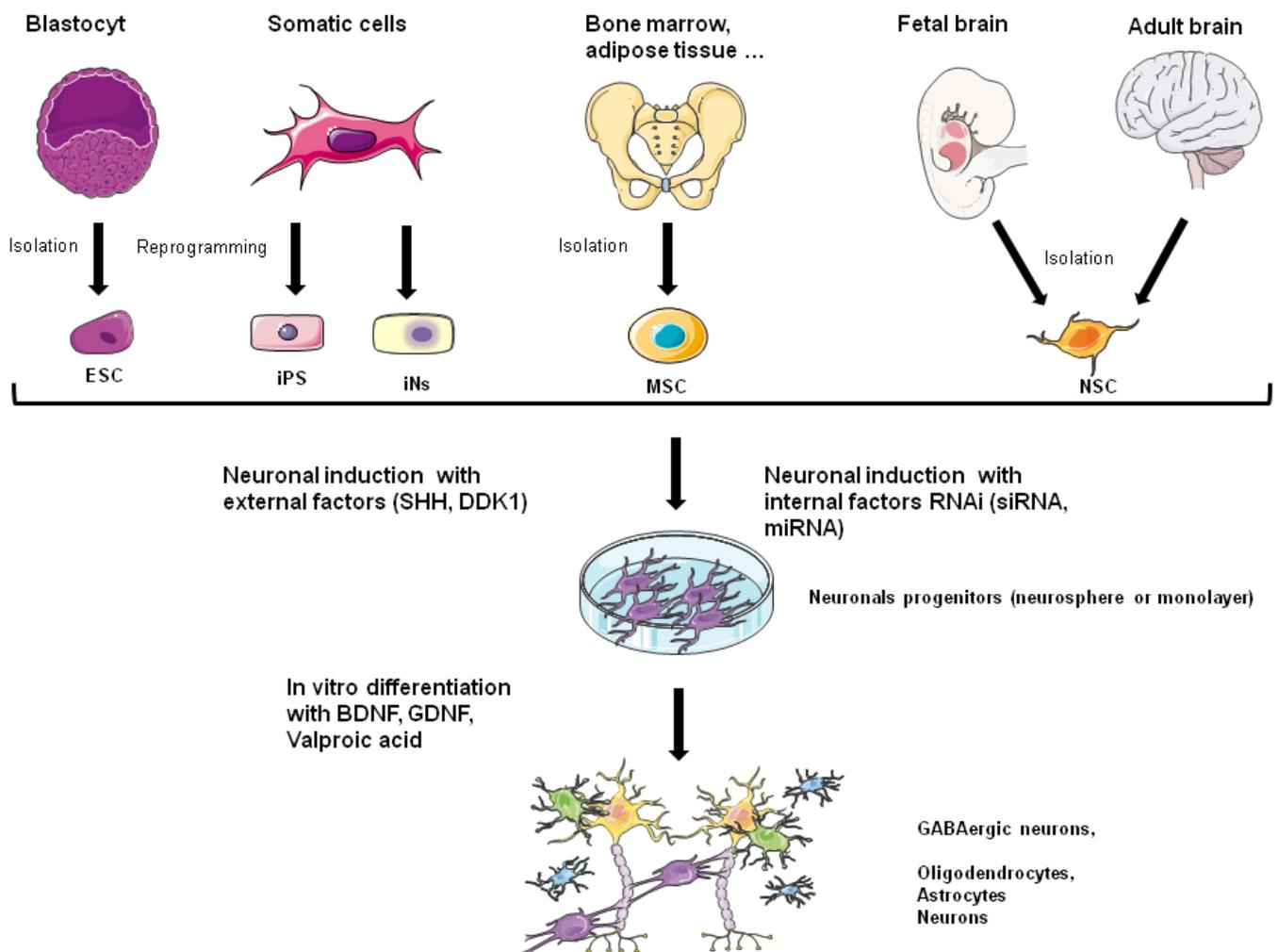
Initially, iPSCs are human somatic cells that are reprogrammed with four retroviral-incorporated specific transcription factors to a pluripotent stem-like state. IPSCs gained immediate international attention for their apparent similarity to embryonic stem cells after their successful creation in 2006 by the group of Yamanaka and in 2007 by Thomson and collaborators [21,22]. Since, many methods have been reported to obtain these cells including high reprogramming efficiency, introducing non-viral and non-integrating methods of cell reprogramming, and using novel gene editing techniques for generating

genetically corrected lines from patient-derived iPSCs, or for generating mutations in control cell lines [23,24]. Moreover, striatal neurons derived from iPSCs obtained from HD patients, provide an *in vitro* disease model of HD. iPSC grafts avoid the ethical issues intrinsic to hESC work whilst also allowing autografts to be performed (for review see [25]). This latter strategy is intuitively more attractive, but does bring with it concerns, particularly in the case of HD patients, that the disease being treated may develop in the grafted tissue given it is derived from the patient themselves. iPSCs may appear to solve the controversy over the destruction of embryos in ESC research, by involving only the genetic reprogramming of somatic cells. However, further analysis of this approach and its subsequent technical and ethical issues such as low reprogramming efficacy, genetic instability, oncogenic potential, conservation of the somatic cell's epigenetic origin, reprogramming process creating totipotent stem cells, could potentially lessen some advantages iPSCs seemingly hold over ESC [26].

Concerning the use of iPSCs *in vitro* for striatal differentiation, the most recent protocol coupled neural induction via Bone Morphogenic Protein (BMP) and Transforming growth factor beta (TGF- $\beta$ ), and ventral telencephalic specification with exposure to the developmental factors SHH and DKK-1, followed by terminal GABAergic differentiation. They obtained a neuronal population monitored by the appearance of progenitors of the LGE and able to mature into GABA- and DARPP-32-positive cells mimicking the striatum ontogenesis in 80 days. These cells presented electrophysiological properties expected for fully functional MSNs. The authors did not explore the iPSC transplantation but studied the outcome of hESC transplantation with their protocol [27]. Results indicated cell survival and extensive axonal projections, suggesting integration of the donor cells into the neuronal network of the host brain with the reduction of motor asymmetry.

For both ESCs and iPSCs, a *bonafide* differentiation protocol, relies on the identification of region and stage specific marker gene determinants of brain development. From a practical standpoint, the general aim is to recapitulate *in vitro* the human foetal development up to the specific and committed neuron precursors of the desired region. The transplanted cell population for HD will

most probably consist of committed LGE progenitors as it has been shown that the highest proportion of striatal-like cells in humans is obtained from grafts comprising the LGE expressing DLX2 under SHH pathways. Further challenges consist in defining the proportion of specific phenotypes that will give the best therapeutic outcome after transplantation, and whether it will consist or not of a population of pure MSN progenitors [28].



**Figure 1. The origin, isolation, & specialization of stem cell to produce neurons in vitro.** The induction of neuronal commitment can be realized by external and internal factors. Recently the combinations of both methods obtain best results.

## 1.4 ADULT STEM CELLS

### 1.4.1 Neural stem cells

An alternative source of cell transplantation in HD would be neural stem cells (NSCs) that participate in normal central nervous system (CNS)

development and differentiate into regionally appropriate cell types in response to environmental factors [29]. In this way, previous studies have shown that NSCs isolated from fetal or adult mammalian CNS can be propagated in vitro [30] and subsequently intracerebrally implanted in animal models of human neurological disorders including HD [31,32]. In the latter case, some cells differentiated in vivo into DARPP32+ neurons replacing neurons primarily targeted in this disorder [31]. Also, genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults [33]. NSCs derived from human brain exhibited extensive migration in the rat brain [34,35] and adult rats receiving intrastriatal transplantation of human NSCs prior to striatal damage induced by 3-NP toxin exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections [36]. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be mediated by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Many studies have also demonstrated that BDNF could block neuronal injury under pathological conditions in animal models of HD [37,38]. Recently, human striatal NSCs were treated with a hedgehog agonist to generate DARPP-32 cells and transplanted in R6/2 HD transgenic mouse brain. The results were disappointing as the outcome was the same as a vehicle control injection. This is the only study using human NSCs for cell therapy in a HD genetic animal model [39].

#### 1.4.2 [Mesenchymal stem cells](#)

Since the discovery by Friedenstein, of colony forming unit fibroblast cells (CFU-F) [40], MSCs never ceased to amaze by the many advantages they have in terms of regenerative medicine. The mechanisms through which MSCs exert their therapeutic potential in tissue repair, although not fully defined, might rely on some fundamental key properties of these cells: i) their ability to secrete soluble factors capable to stimulate survival and functional recovery of injured cells; ii) the ability to home to sites of damage; iii) the ability to modulate immune responses and iv) their easy accessibility and potential for autologous transplantation. Although these cells differentiate to mesodermal lineage

(chondrocytes, adipocytes, osteoblasts) differentiation of MSCs toward non-mesodermal cells has also been reported (for review see [41]).

MSCs may be expanded *in vitro* with varying degrees of additional differentiation towards neuronal lineages [41,42] enabling their use for cell therapy for neurodegenerative disease including HD. MSCs are a heterogeneous population with cells presenting different differentiation properties and capable of expressing specific embryonic markers. A homogenous subpopulation of MSCs, named “Marrow-Isolated Adult Multilineage Inducible” (MIAMI), which express several pluripotency markers (Oct4, Sox2, Nanog, SSEA4) are able to generate cells derived from all three embryonic germ layers [43]. They are capable of differentiating into neuronal-like cells with electrophysiological properties of immature neurons under the influence of a sequential addition of specific growth factors and respond to dopaminergic inducers acquiring this neuronal phenotype [44,45]. Moreover, they also show some degree of *in vivo* neuronal differentiation and they can secrete high amounts of tissue repair factors, some of which are involved in the protection/induction of blood vessels *ex vivo* [46]. Recently, adult human somatic cells, such as MSCs and dermal fibroblasts were shown to contain a small number of severe stress-tolerant pluripotent stem cells, named “multilineage differentiating stress enduring” (Muse) cells [47]. These cells expressing pluripotency markers such as Nanog, Oct3/4, and Sox, can be isolated from MSCs or from fibroblasts after severe stress as stage-specific embryonic antigen-3-positive cells (a marker for human ES cells). Most importantly, they possess specific properties like self-renewal, and pluripotency as they are able to generate cells representative of all three germ layers from a single cell. One limitation to the use of Muse cells is linked to their low yield (with the range between 1% – 5%), but their stress-resistance makes of them an interesting cell therapy candidate [47].

These cells are implanted either non-differentiated or after a pre-differentiation stage, as precursors, relying on the host environment to drive selective functional differentiation for cell replacement. However, the lack of consistent neuronal differentiation of transplanted MSCs has limited their therapeutic efficacy in animal models of HD (**Table 1**). *In vitro* and *ex vivo* characterizations of MSCs have revealed the presence/secretion of many growth

repair factors, including BDNF, Glial cell-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) [48–50]. Researchers have recently explored the secretome of MSCs, with the aim of identifying all secreted molecules, which, in turn, may provide insight into the mechanisms of MSC benefits (for review see [51]). Indeed, intra striatal transplanted MSCs integrated in the host brain and exerted neurotrophic effects that correlated with increased levels of laminin, von Willebrand factor (VWF), stromal cell-derived factor-1 (SDF-1)  $\alpha$ , and its receptor in the damaged striatum [52]. Moreover, after MSCs transplantation into the QA model of HD, rats presented a significant improvement in apomorphine-induced rotation tests, which correlated with a reduced lesion volume and a lower number of apoptotic striatal cells compared to control animals [53]. In the R6/2 mouse HD model MSCs transplanted mice showed improved motor functions compared to untreated R6/2 controls, although the overall performance continued to decline. Interestingly, mice, which received MSCs transplantation, displayed a significant longer survival time than untreated R6/2 mice [54,55]. The improvement may be imputed to BDNF secreted by MSCs into the striatum. Moreover, the secretion of BDNF has been associated with recruitment of forebrain neural progenitors [56][57]. MSCs pre-differentiated towards a neuronal phenotype, in order to better engraft within the brain parenchyma, may also be able to release the required repair factors involved in neuroprotection. One study showed that the specification of MIAMI cells towards a neural/early neuronal phenotype still enabled the secretion of neurotrophic factors by these cells [45], but the appropriate degree of commitment before transplantation needs to be investigated.

Transplanted cells	Animal models	Mechanism	Functional outcome	References
Adipose MSC	Rat, QA	Increase CREB, PGC1 <sub>alpha</sub> , reduced apoptosis	Slowed striatal Degeneration and decreased lesion volume	[55]
Bone MSC	marrow Mice, QA	SDF1 and VWF secretion, reduced apoptosis	Improved survival Decrease lesion volume Improved rotared performance	[58]
Bone MSC	marrow R6/2- mice	SDF1 secretion, Von Willebrand factor (VWF) secretion, reduced apoptosis	Improved survival	[58]
Bone MSC	marrow Rat, QA	Expressed BDNF, NGF, GDNF, CNTF	Decreased lesion volume Improved rotared performance	[59]
Adipose MSC	R6/2- mice	Reduction HTT aggregates Increase PGC1 <sub>alpha</sub> , Akt/cAMP expression	Improved rotared performance Improve striatal volume	[55]
Adipose MSC	YAC 128 mice	Increased BDNF, HGF, IGF, LIF, and VEGF expressions	Improved rotared performance Motor function Improved striatal volume	[54]
Bone MSC BDNF	marrow producing YAC 128 mice	Both BDNF-modified and non- modified MSCs had significant effects in reducing the behavioral defects in an HD mouse model.	Improved rotared performance Reduced hindlimb clasping	[60]

**Table 1. Human mesenchymal stem cells based treatment of Huntington's disease in preclinical animal's model.**

### 1.4.3 Induced neuron cells

It has recently been shown that mouse dermal fibroblasts can be directly programmed to functional neurons by forced expression of a set of neural lineage transcriptional factors, named induced Neurons cells (iNs) (for review see [61]). The authors initially tested a pool of 19 transcription factors from which three (Ascl1, Brn2 and Myt11, abbreviated hereafter as BAM factors) were found sufficient to generate functional neuronal phenotype from fibroblasts [62]. Several different groups, after reprogramming fibroblast from

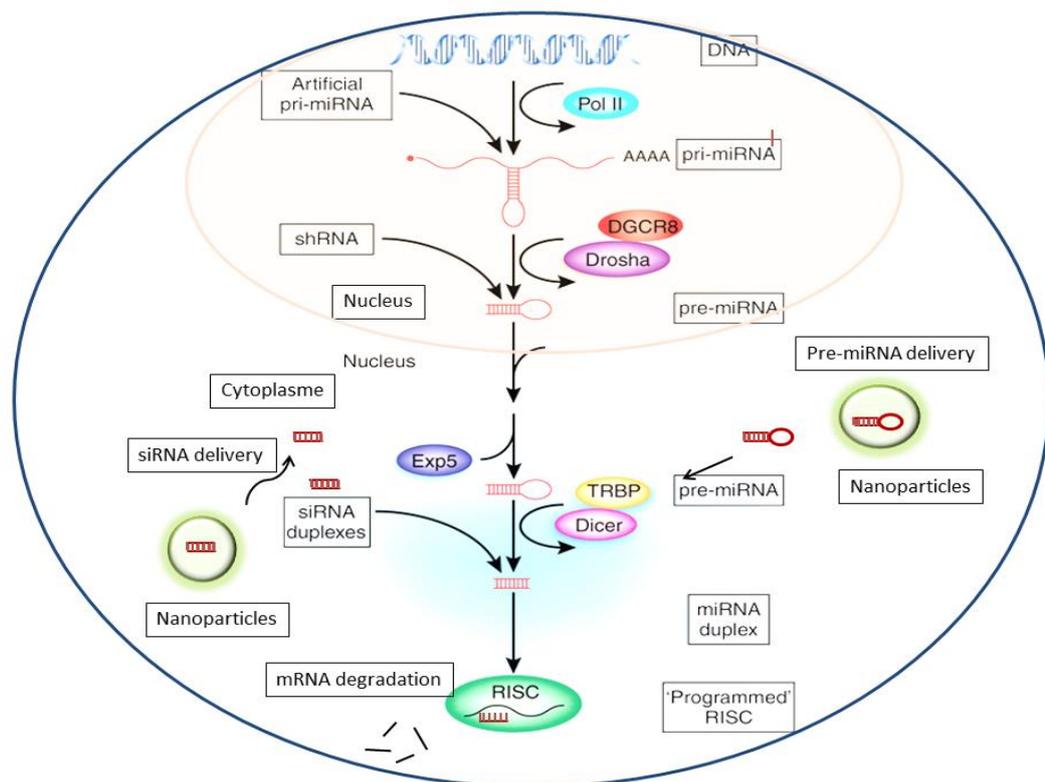
mouse, reported the generation of human iN cells (hiNs) [63–65]. None of the groups were successful in producing functionally mature hiN cells using the same three transcription factors (BAM) that worked in mouse cells [65]. A major limitation of direct conversion to a terminally differentiated mature cell type is the inability to expand the programmed cells in sufficient quantity for various applications [66]. Therefore, direct conversion to expandable neural stem/progenitor cells (NSCs) is desirable in practical applications that demand large amount of cells.

## **2. EPIGENETIC MANIPULATION**

As described, cells may be “manipulated” to control their proliferation, to improve their survival and differentiation and therefore ameliorate their engraftment after transplantation. This can be achieved by a previous in vitro genetic or epigenetic manipulation of the transplanted cells or by co-transplanting them with transcriptional and/or trophic factors that can be delivered by vectors and biomaterials. The latter being moreover able to provide a 3D template of extracellular matrix (ECM) contributing to shape the cells into a functional tissue and increase their engraftment [17,45,19,27]. The real challenge consists in controlling stem cell survival and differentiation by monitoring intracellular levels of relevant biomolecules while replacing lesioned tissues with these cells. Moreover, if possible, they should maintain their neuroprotective effects, when present. Typically, cell programming (differentiation within the same lineage) or cell reprogramming (reversal to pluripotency) consists in overexpression or knocked-down expression of genes involved in stem cell differentiation or transdifferentiation (passage to a different lineage) in a specified manner. The control of gene expression may be achieved by RNAi, by selectively knocking down the expression of only a few pivotal genes. This epigenetic modification is quite simple, does not need genetic manipulation, is transitory and is now quite well understood.

RNAi were first used in 2003 to promote differentiation to the myogenic lineage from pluripotent P19 teratocarcinoma [67]. Differentiation to the neuronal lineage is one of the most popular areas in cell therapy due to the poor regenerative potential of the nervous system. In fact, a large number of studies

have been dedicated to identifying potential knockdown targets to facilitate neurogenesis in ESCs, NSCs and MSCs. For example, the use of a lentiviral-mediated RNAi vector that down-regulates the expression of REST (repressor element-1 silencing transcription factor) was shown to promote MSC differentiation into neuronal cells [68]. The unrestricted potential of RNAi has encouraged strategies for large scale silencing of protein encoding genes in the human genome. RNAi can be triggered by three different pathways: 1) a RNA-based approach where the effectors siRNAs are delivered to target cells as preformed 21 base duplexes; or 2) a DNA-based strategy in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts; 3) a RNA-based approach, similar to siRNAs, produced endogenously by the cells, the pre-miRNAs stem-loops which are then spliced to release the mature miRNA duplex (**Figure 2**).

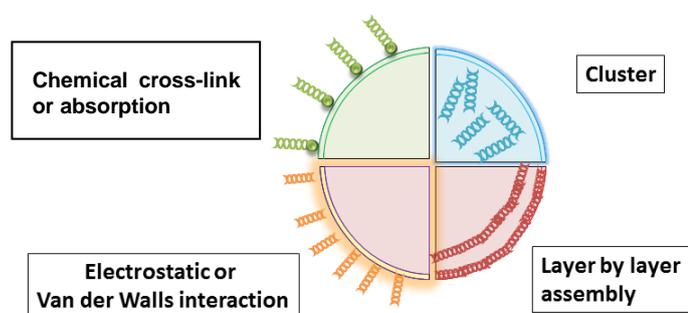


**Figure 2.** Inspired from Cullen, *nature genetics* 2005, we represent the intracellular trafficking of siRNA delivery systems [69]. Internalization of delivery systems can be performed by several mechanisms as clathrin, caveole pathways or lipid rafts. Two modalities are observed in this phenomenon, a receptor mediated endocytosis and a non-receptor mediated endocytosis. RNAi is delivered into the cytoplasm and produces its inhibitor effect.

*In vitro*, RNAi does not readily bind to or cross the cell membrane, so RNAi must be introduced in cells by innovative delivery systems. In the case of

RNAi, the siRNA or miRNA must be delivered to the RNA-induced silencing complex (RISC) in the cytoplasm (**Figure 2**). The most common delivery system relies on recombinant virus, but although very effective, viral methods for RNAi delivery have been associated with immunogenicity and tumorigenicity [70]. Non-viral delivery systems are traditionally less effective but can be designed to avoid issues typical of viruses. They have considerable advantages over viral-based vectors due to the control of their molecular composition, their simplified manufacturing, modification and analysis, and their tolerance for cargo sizes, while displaying a relatively low immunogenicity [71]. Non-viral gene delivery utilizes chemical reagents including lipids, cationic polymers and nanoparticles or physical means such as electroporation. More interestingly, some nanocarriers can secure the control of cellular behaviour. Indeed, materials with unique nanotopographical characteristics and size offer properties producing similar effects than growth factors [72]. They can be used to induce specific biological responses. However, before RNAi reaches its target *in vitro* and *in vivo* when co-transplanted with delivery vectors, it faces a number of significant barriers. More importantly, cellular and local delivery strategies have to deal with the need for internalization, release, and distribution in the proper subcellular compartment (**Figure 2**). Many early efforts at RNAi delivery used materials that were already well studied for DNA delivery and can be applicable, with some limitations, to the RNAi delivery. Development of different strategies to encapsulate and deliver RNAi has been described in literature [73] (**Figure. 3**). We will first discuss the properties of these nanocarriers, which make them suitable for nucleic acid delivery in general and then describe their utility for overcoming barriers specific to RNAi delivery and their use in stem cells for controlling neuronal differentiation or survival.

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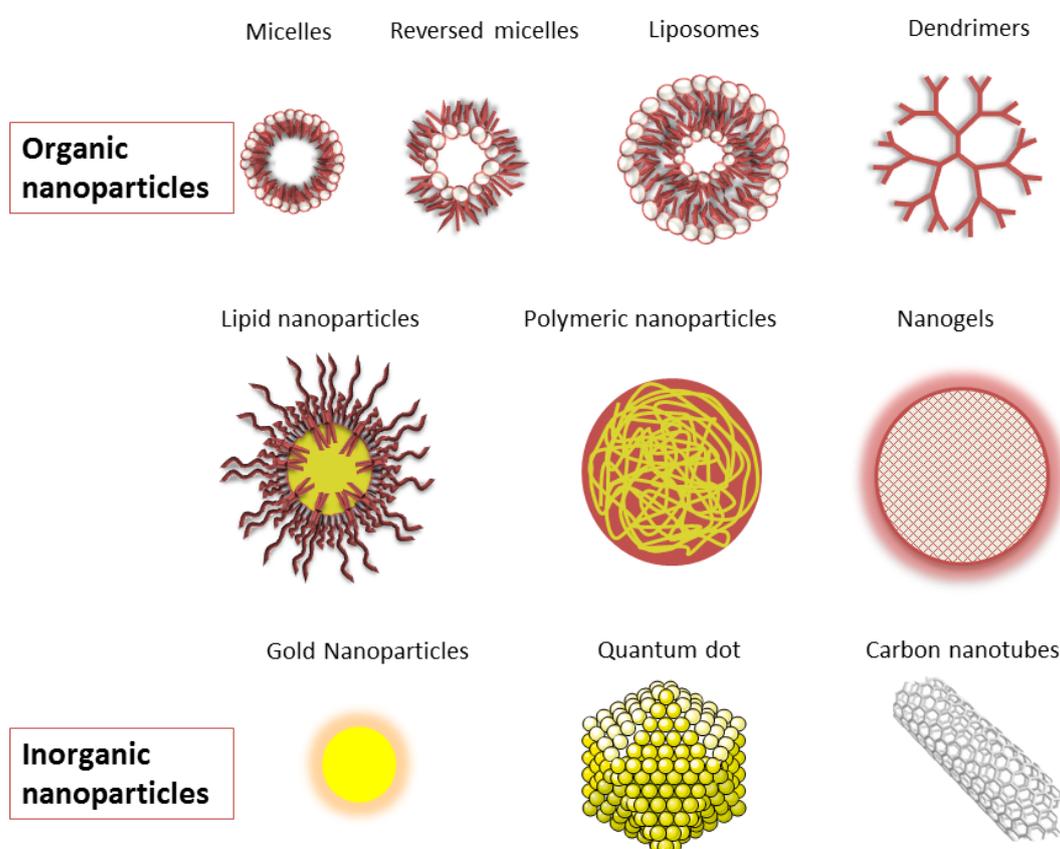
**Figure 3. Methods of RNAi complexation with delivery systems.** The RNAi localization into nanocarriers differs according to structure and organization of the delivery system. RNAi can be incorporated into a matrix structure and allow a global repartition in the entire volume of the sphere (cluster), with polymers components. Other strategies consist in either associating by electrostatic or Van der Waals interactions the RNAi on the surface of delivery systems. The chemical cross-link is also a possibility. Last organization concerns shell/core structure observed for liposomes and some nanoparticles. [74].

## 2.1 NANOCARRIERS TO CONTROL STEM CELL FATE

The use of nanocarriers, also named nanoparticles (NPs) ranging in size from 1nm to 1000nm to control stem cell fate after intracellular incorporation is a novel approach. NPs were first developed for the potential delivery of therapeutic factors such as chemotherapeutic agents to tumours or, when combined to stem cells, mostly for stem cell imaging [71]. The nanocarriers most commonly used in stem cell research are organic (liposomes, lipid nanoparticles, solid nanoparticles and dendrimers) and inorganic nanocarriers (quantum dots, carbon nanotubes, iron or gold nanoparticles) (**Figure 4**). These last materials with unique characteristics such as carbon nanotubes, gold nanoparticles, and gold nanorods have attracted attention as innovative carriers for RNAi (for review see [75]) but have not yet been used for cell programming *in vitro* combined with RNAi. However, inorganic nanoparticles seem to possess certain properties stimulating neuronal differentiation. Iron oxide nanoparticles incorporated within rat PC12 cells induced extensive changes in genes related to the cytoskeleton, signalling molecules, receptors for growth hormones and ion channels, all required and expected to occur during neuronal differentiation [76]. One explanation for the effect on gene expression is that several inorganic particles, including iron oxide nanoparticles, have the potential to release inorganic ions. In the case of manganese, the ions play an important role in neuronal differentiation by activating cell adhesion molecules, which interact

with the extracellular matrix and direct cell binding and signalling. Neuronal differentiation is known to be influenced by the amount and subcellular distribution of integrin clusters, i.e. cell adhesion [77]. However, the mechanism by which metal ions affect gene expression is still unclear.

The encapsulation of hydrophilic drugs such as nucleic acids and also proteins has been developed from different formulations. A large variety of synthetic materials or polymers, such as polylactide-*co*-glycolide (PLGA), polycaprolactone, and natural materials, such as chitosan, collagen, pullulan, and poly-arginine can be used to formulate nanoparticles for medical applications.



**Figure 4.** Different types of nanocarriers able to control the stem cell fate. Nanoparticles are commonly defined as objects with a range of dimensions of 1-1000nm, which includes micelles, reversed micelles, nanoparticles, nanogels, and nanofibers. We represent here the morphology of the most commonly particles used ones for siRNA complexation and regenerative medicine.

## 2.2 LIPID-BASED NANOCARRIERS

Lipid-based nanocarriers are the most widely used biomaterials for nanoparticulate RNAi delivery. Of over 20 siRNA phase I clinical trials, nearly

half use NPs as the delivery vehicle, and almost all of these are lipid-based [78]. Although lipid-based nanoparticles have been historically designed for lipophilic drug delivery, the idea of using cationic lipids with their positive charged head group to efficiently bind negatively charged RNAi became rapidly evident.

### 2.2.1 [Liposomes](#)

Transfection typically involves the use of packaging particles called liposomes to facilitate the cellular uptake of RNAi. Cationic lipids in the liposomes mimic the physical characteristics of natural phospholipids that represent the individual components of a cellular membrane. Liposomes possess long hydrophobic chains and a positively charged head group, allowing the formation of nano-sized complexes with negatively charged RNAi (lipoplexes) that is encapsulated by a lipid bilayer. Since first being used for gene therapy in 1990, numerous commercial cationic lipids (also called cytofectins, lipofection, oligofection reagents) have been synthesized and used for delivery in cell culture [79]. Lipoplexes offer protection to RNAi from enzymatic degradation and efficient endocytosis of RNAi by the cell. Lipid complexation with the payload (i.e. RNAi) simply involves mixing and incubation [71]. Neutral lipids such as dioleoylphosphatidylethanolamine (DOPE) and cholesterol are usually incorporated in the liposomal formulations, where they serve as helper lipids: increasing the transfection efficiency of the gene or molecule-containing liposome by facilitating membrane fusion and aid in the destabilization of the plasma membrane [80]. Liposomes can escape from the endosome through a so called ‘proton sponge’ effect where positively charged cationic lipids cause influx of protons and water leading to endosome swelling and eventually disruption to release the lipoplexes to cytoplasm. Safe and efficacious delivery *in vivo* is however rarely achieved due to toxicity, nonspecific uptake, and unwanted immune response.

Very few studies however transfect human iPS or ESC cells with RNAi to drive neuronal differentiation because limited transfection efficiencies have been reported in this type of cells and transitional action reported with these materials is not sufficient on their own to have an effect. Indeed, *in vitro* transfection efficiency is affected by the type of cell, proliferative activity of

the cell, the type of culture media and cell plating density. It appears that lipoplexe transfection performed well in human MSCs [81,82], but produced more inferior outcomes in NSCs [83] and ESCs [84,85]. These results suggest that conventional lipoplexe transfection is an efficient RNAi delivery means for human MSCs but not for human ESCs. In order to determine guidelines to transfect human ESCs while inducing their differentiation, two groups have attempted to knock down Oct4, a pluripotent stem cell gene, in ESCs with transfection efficiencies of only ~60% [84] and 69.8% [85] compared to Lin28, another pluripotent marker. This low efficiency for Oct-4 suggests that the type of target molecule will also affect the transfection outcome. Interestingly, the transfection efficiencies and the resulting silencing efficiencies were not strictly proportional to each other; with a more efficient Oct4 knock down being obtained by the less effective transfection. This is probably attributed to auto-regulation mechanisms of the levels of Oct4, which were moreover quickly restored after 1 day, indicating the need for additional boosters to further increase the silencing efficiency to >90%.

Cationic lipids were the first non-viral vectors demonstrating their capacity to easily transfect mouse embryonic stem cells *in vitro* with microRNAs (miRNA) in order to induce neuronal commitment [86]. In order to determine the role of miRNA-125b mimic in human MSC during *in vitro* neuronal differentiation, the cells were transfected or lentiviral infected with miRNA-125b resulting in the increased percentage of TH-positive neurons. With the two methods used, similar results were obtained confirming its role in dopaminergic differentiation. The miRNA-181\* also increases the number of TH-positive neurons, intriguingly, just one of the strands allows this differentiation [87]. Furthermore, the role between miRNA 9 and REST to promote neuronal commitment was determined. A mutual down-regulation between REST and miRNA-9 was observed, which may contribute to the maintenance of a neuronal differentiation program [88]. More interestingly, MSCs after liposomal transfection with miRNA-124 and miRNA-145, are able to deliver exogenous miRNAs to human NSC in co-culture with astrocytes via exosomes. Moreover, the delivered miRNAs altered gene expression in the recipient neural cells and impacted their phenotype and function. The delivered miRNA124 increased the expression of the glutamate transporters,

EAAT1/EAAT2 in NSC and astrocytes and down-regulated Sox9 expression which increased the expression of  $\beta$ 3-tubulin, a marker of neuronal precursors [89]. Similar results were obtained with MSCs transfected with pre-miRNA-124. MSCs can therefore be easily transfected with exogenous miRNA to further induce neuroprotection or the differentiation of neuronal progenitors after transplantation.

The transfection efficiency with these delivery vehicles *in vitro* is, in part, determined by their stability and particle size. For example, serum proteins can decrease transfection efficiency by neutralization of the positive zeta potential [90] of the complexes. Toxicity may, in part, result from the large size of the complexes, and the high positive zeta potential required for their uptake [91]. The toxicity is normally closely associated with the charge ratio between the cationic lipid species and the nucleic acids, as well as the dose of lipoplexes administered. So although cationic lipid formulations have been used to deliver RNAi *in vitro* and *in vivo*, cell toxicity caused by cationic lipid is still a major concern. To bypass problematic toxicity, particle instability and to maximize siRNA delivery, fragments of liposomes or lipoplexes can be encapsulated into nanoparticles.

### 2.2.2 [Lipid based nanoparticles](#)

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) consisting of spherical solid lipid particles in the nanometer range are an interesting alternative to liposomal toxicity. They are comparatively stable colloidal carrier systems in which melted lipids are dispersed in an aqueous surfactant by high-pressure homogenization, solvent injection, solvent emulsification or micro-emulsification [92]. The lipids which are used in their production are solid at room temperature, and most of them have an approved status, e.g. GRAS (Generally Recognized As Safe), due to their low toxicity [93]. Although cationic lipids are an inherent part of SLN formulations, it is unclear to which extent their presence bears relevance to the mechanism by which SLNs deliver their cargo. Whether their polymorphic properties play a role in destabilizing cellular membranes, as discussed above for cationic lipid-based nanocarriers, remains to be determined. *In vitro*, the comparison of cell time retention of siRNA between SLN, lipofectamine<sup>®</sup> and liposomes showed

that the siRNA is present during five to nine days for SLN and three days for commercial reagents or liposomal formulations. This property is quite interesting for maintaining a differentiated cellular state over time for cell therapy applications. However, the percentage of siRNA loading is quite low around 35% and still needs to be improved [94]. Nanostructured lipid carriers possess the advantage of offering increased hydrophilic drug loading and decreased drug leakage from the NPs.

Lipid nanocapsules (LNCs) came into the spotlight as a strategy to overcome positively charged lipoplexe toxicity encapsulated within the carriers, while enhancing cell uptake and protecting the nucleic acid from lysosome degradation. Our research group has reported innovative LNCs, consisting of a lipid liquid core of triglycerides and a rigid shell of lecithin and polyethylene-glycol. The simple solvent-free formulation process based on the phase inversion of an emulsion makes them an ideal nanocarrier for translational studies. They are highly stable (more than 3 months) [74] in comparison to SLNs [95] and, in function of their composition and size, can escape the endo-lysosomal compartment [96]. These LNCs can mediate highly efficient gene transfer not only *in vitro* but also *in vivo* [97,98]. They were recently modified to encapsulate siRNA, complexed with DOTAP/DOPE lipids forming lipoplexes (Fig. 3), and showed efficient encapsulation and protein inhibition in a glioma cell-line [98]. Interestingly, the cellular uptake mechanism with siRNA-LNCs seems to be membrane fusion. The study of this mechanism by Fluorescence confocal spectral imaging demonstrated the degradation of this nanocarrier and the release of siRNA in the cytoplasm explained beside by the presence of DOPE lipids in the formulation [99]. LNC-siRNA transfection for neuronal commitment and resulting protein inhibition capacity is currently being evaluated in MSCs.

Recently, our research group has reported a novel lipid nanocarrier based on sorbitan monooleate, more commonly known by the commercial name of Span 80 (SP80); a surfactant widely used in the pharmaceutical industry and generally recognized as safe (GRAS). However, instead of using span as a surfactant, this component was used as a lipophilic main ingredient of these nanosystems which have demonstrated different component loading capacities

(Sanchez et al., WO2013068625A1) [100]. This new system can incorporate cationic components such as oleylamine or poly-arginine to complex RNAi by electrostatic interaction at the surface (Fig. 3). The capacity to load a variety of polymers into the shell offers an adaptable system for all the types of cells and the possible incorporation of natural polymers can reduce the cytotoxicity.

## 2.3 POLYMER BASED NANOCARRIERS

Polymer based nanocarriers are a very promising class of biomaterials for the delivery of nucleic acids and are an active subject of ongoing research. Because many of the polymers used for RNAi delivery were originally investigated as DNA delivery materials, generalities about DNA delivery can be extended to RNAi delivery. Polymers used for RNAi delivery can be divided into two categories: (i) those with synthetic components, such as dendrimers, polyethylenimines (PEI), etc (ii) those with biodegradable natural components, such as collagen and pullulan. We will briefly describe most synthetic polymers used for RNAi delivery for stem cell neuronal differentiation and then we will present novel nanoparticles with natural polymers.

### 2.3.1 Synthetic components

PEIs, presented in branched or linear forms, available in a broad range of molecular weights are widely used for gene delivery. In the physiological medium, PEI is positively charged due to protonation of the amine groups and thus can be used to condense nucleic acids. [101]. Cationic polyplexes, formed by PEI and nucleic acids, typically retain a net positive charge promoting interactions with negatively charged polysaccharides on the cell surface followed by endocytosis. The polyplexes escape from the endosome by the same 'proton sponge' effect described for cationic liposomes. PEI is known to mediate highly efficient gene transfer, but also exhibits significant toxicity during the transfection process [102]. Therefore, many studies have reduced the cytotoxicity caused by PEI-mediated delivery by conjugating PEI to synthetic or natural polymers, such as hyaluronic acid [103]. In this way, several studies have shown that PEI-modified polymers can be used to deliver genes to regulate stem cell differentiation usually with DNA but also in combination with siRNA [104]. In this regard, combined transfection of a Sox9-bearing plasmid and

Cbfa-1 siRNA complexed with PEI–PLGA nanoparticles were found to enhance hMSC chondrogenic differentiation [105]. To our knowledge, no neuronal stem cell differentiation study has yet been performed with these polymers. Nevertheless, the inhibition of ROCK (with PEG–PEI/siROCK2) increased the neuroprotection against an external agent of primary cortical neurons [106]. Despite these advances in PEI-based gene delivery, it remains a challenge to balance the transfection efficiency and cytotoxicity of PEI-based delivery systems.

Dendrimers have attracted a great deal of interest in different areas including nucleic acid delivery applications for cell differentiation due to their uniform, well-defined, three dimensional structures [107]. The name "dendrimer" originated from the greek words which describe a structure consisting of a central core molecule that acts as a root, from which a number of highly branched, tree-like arms originate in a well-ordered and symmetrical manner [107]. The commercial dendrimers named PAMAM transfection reagents (Superfect and Polyfect) are mainly indicated for plasmid DNA and RNAi transfection. Amine-terminated PAMAM dendrimers develop highly positive charged densities at their surfaces when they are at physiological pH or dissolved in water. However, double stranded RNA including siRNA and miRNA are less flexible than pDNA, which can lead to the incomplete encapsulation or the formation of undesirably large complexes. Highly branched dendrimers have thus been developed for siRNA efficient delivery [108,109]. Different strategies have been further developed to reduce the toxicity of dendrimers, such as the neutralization of the surface charge with PEG, the attachment of peptides at the surface [110], or the association with natural components such as collagen [111]. PAMAM, with a reported ability to trigger a proton sponge endosomal escape, showed an efficient delivery of RNAi in neurons *in vitro* and *in vivo* (intracranial injection in rabbits) with very low toxicity levels [112]. Interestingly, the silencing of high mobility group box-1 (HMGB1, a novel cytokine-like molecule) in primary cortical cultures successfully reduced both basal and H<sub>2</sub>O<sub>2</sub>- or NMDA-induced neuronal cell death [113]. A similar approach could be tested with neuronal committed stem cells to increase their survival after transplantation in HD paradigm.

Nowadays, the combination between siRNA and morphogens are effective for stem cell differentiation. Shah et al. (2013) developed cyclodextrin-modified dendritic polyamine construct (termed DexAM) for effective stem cell differentiation [114]. DexAM present the ability to simultaneously deliver nucleic acids (siRNA against Sox9) and hydrophobic small molecules (Retinoic acid) to achieve a synergistic enhancement in stem cell differentiation. When rat NSCs were transfected with siSox9 using these nanoparticles, they differentiated into neurons, wherein more than 70% expressing the neuronal precursor marker B3-tubulin [114].

### 2.3.2 Natural components:

Recently, siRNA-grafted natural polymers have been proposed as a promising strategy for siRNA delivery. They can be divided into two main categories: (i) polymers obtained strictly from natural sources, (ii) and semi-synthetic polymers obtained from natural sources but which are rationally modified with the aim of adapting their properties according to the delivery requirements of the therapeutic molecules.

Chitosan has been widely described in the formulation with RNAi because of its cationic nature, low toxicity, biocompatibility, and degradability in the human body and has been designated as safe (GRAS) by the FDA [115]. This linear polysaccharide of randomly distributed *N*-acetylglucosamine and glucosamine units is protonated in slightly acidic conditions, which permits an efficient complexation of nucleic acids into NPs. Recently, a commercial DNA transfection reagent based on a chitosan oligomer has been developed (Novafect, Novamatrix). Regarding siRNA delivery, various studies describe the importance of chitosan characteristics to achieve good efficiency *in vitro*, including the degree of deacetylation and molecular weight (MW) of the chitosan, charge ratio of amine (chitosan) to phosphate (RNA), serum concentration, pH and cell type [116]. However, this system has a significant limitation, owing to its low transfection efficiency [117].

Spermine had been recently shown to enhance transfection when grafted in the anionically modified pullulan exhibiting excellent blood compatibility and *in vitro* transfection. More interestingly a complexation to pullulan–spermine

achieved successful *in vitro* neuronal gene delivery irrespective of the negatively charged complexes with no measurable cytotoxicity at up to 20 µg/ml DNA [118]. Actually, the tendency to improve transfection efficiency and reduce the cytotoxicity is mixing natural components with lipids, or inorganic components to obtain hybrid systems [119].

Although many efforts have been made to drive cells into specific lineages, maturity and functionality remains a problem for regenerative medicine. Most probably, two dimensions (2D) culture do not authorize a complete and functional differentiation. Number of publications reporting benefits on three dimensions (3D) culture is increasing, and 3D supports have been demonstrated as improving cell survival after grafting. Micro- and nano-scale chemical and physical cues from the ECM environment control and direct various key cell behaviours including their adhesion, proliferation, migration and differentiation.

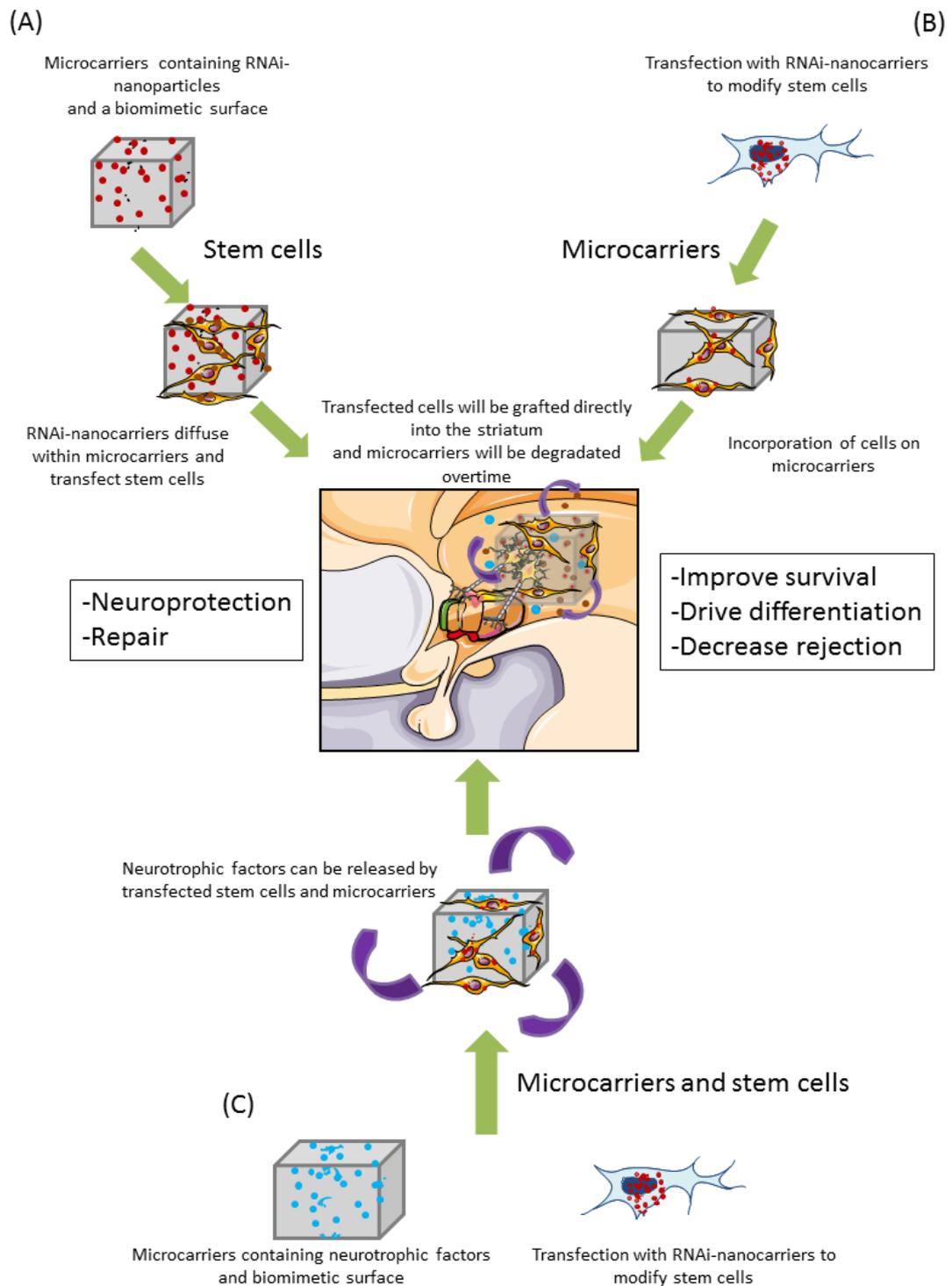
### **3. COMBINED STRATEGIES FOR CELL TRANSPLANTATION**

Strategies to achieve brain protection in HD, repair and recovery include the delivery of neuroprotective compounds to prevent cellular degeneration, cell transplantation to replace lost cells, approaches using tissue engineering, and methods to enhance plasticity by promoting the intrinsic capacity of the brain to regenerate and reorganize. The latter strategy still has poor control of cellular growth processes, differentiation and migration to the appropriate location *in vivo*. Cell-based therapies have encountered poor cell survival and integration in the host. Moreover, it is necessary to recapitulate the sophisticated and precise architecture and functional wiring present in the cellular and molecular environment of the brain. Therefore, combined *in vivo* strategies using stem cells, biomaterials, growth factors and epigenetic control of gene expression with different vectors are nowadays being investigated (**Figure. 5**).

#### **3.1 CELL AND TROPHIC FACTOR RELEASING MICROCARRIERS**

Transplanted stem and progenitor cells can promote the survival of host cells by releasing neuroprotective trophic factors. In addition, many studies have demonstrated the preclinical feasibility of encapsulation as a means of

delivering factors to the CNS, and more particularly, genetically engineered cells secreting trophic factors for HD [120]. In general, these studies demonstrate that encapsulated cells can be protected and remain viable for extended periods of time to produce significant neuroprotective and behavioral benefits. Clinical trials have been conducted on evaluating the benefits of encapsulated cells delivering ciliary neurotrophic factor for HD [121]. In this case, the implanted cells were safely tolerated without serious adverse events, justifying further clinical evaluation. However, the relatively modest cell survival and the heterogeneity of the results in these studies need to be improved.



**Figure 5. Schematic illustrating technologies to spatially control stem cell fate.** During microcarriers' process, nanocarriers containing RNAi or neurotrophic factors can be integrated directly into these biomaterials. All possibilities for combined strategies for stem cells transplantation have been presented. The first consideration for microcarriers tailoring in brain is size: microcarriers should necessarily be small enough to be easily implanted into the striatum, either via stereotactic implantation or under neuro-navigation. Moreover, small-sized scaffolds render repeated implantations possible, with no need for open-surgery [122].

Another way to deliver a growth factor is after encapsulation within polymeric microcarriers that in their turn may transport cells on their surface (for review [123]). In this sense, our group has developed the pharmacologically active microcarriers (PAMs) combining these two approaches. These PAMs are biodegradable and biocompatible PLGA microspheres conveying cells on their biomimetic surface, therefore providing an adequate 3D microenvironment *in vivo*. Moreover, the controlled delivery of a trophic factor in combination with a biomimetic surface acts synergistically to stimulate the survival and/or differentiation of the grafted cells toward a specific phenotype, therefore enhancing their engraftment after their complete degradation [124]. Finally, it should be noted that the delivered molecule might also affect the host microenvironment allowing the integration of the grafted cells and/or stimulating the lesioned brain repair capacities. The efficacy of PAMs for cell therapy of Parkinson's disease in a clinical paradigm was demonstrated using GDNF-releasing PAMs, conveying a small number of embryonic ventral mesencephalon dopaminergic cells [44]. Similarly, PAMs with a biomimetic laminin surface delivering Neurotrophin factor-3 and conveying adult stem cells showed a neuroprotective and repair effect leading to an ameliorated behavior in an animal model of Parkinson's disease [125]. This technology can be adapted for HD, delivering stem cells and neurotrophic factors able to drive GABAergic commitment such as BDNF. However, as described, external factors (media and growth factors) are insufficient to significantly control stem cell fate on their own; the ideal strategy relies on their combination with the modification of gene expression by RNAi (Fig. 5).

Another study demonstrated enhancement of neuronal differentiation of NPCs by nanofibrous carriers-mediated release of BDNF and retinoic acid (RA). In that study, nanofibrous were constructed with the copolymers of  $\epsilon$ -caprolactone and ethyl ethylene phosphate. BDNF and RA were then encapsulated in the carrier. Neuronal differentiation of NSCs was enhanced due to the synergistic effects of nanofibrous topography and sustained delivery of BDNF and RA [126]. These cell-loaded capsules can be implanted into the damaged brain area favoring the local, targeted and long-term release of drugs or proteins.

### 3.2 RNAI LOADED NANOCARRIERS AND MICROCARRIERS FOR REGENERATIVE MEDICINE

Conventional approaches using cationic polymers or lipids usually only permit transient gene expression in stem cells after transfection [127]. To overcome the limitation of gene delivery using only cationic substances, porous 3D carriers made of biodegradable polymers, such as sponges, particles, or hydrogels, can be employed as gene carriers and depots for prolonged gene delivery. Immobilization or incorporation of gene (DNA, RNA) complexes with cationic polymers or lipids in the carriers may facilitate sustained delivery to stem cells cultured on the scaffolds, leading to prolonged transgene expression in the stem cells and extended control of stem cell differentiation [128].

MSCs osteogenic differentiation was enhanced by decorated nanostructured scaffold, composed by poly- $\epsilon$ -caprolactone capable of retaining and delivering siRNA, with broad applications for controlling stem cell differentiation *in vitro* and *in vivo*. [129]. Recently, a simple and efficient siRNA delivery system based on nanotopography-mediated reverse transfection was developed. The authors deposited a self-assembled silica nanoparticle monolayer on a glass cover slip and then coated extracellular matrix proteins and siRNA on top of the nanoparticle monolayer [128]. This platform allowed highly efficient transfection of Sox9 siRNA into rat NSCs due to nanotopographical cues for stimulating endocytosis and intracellular gene transfer, which enhanced neuronal differentiation of NSCs while inhibiting glial differentiation [128]. Low et al. (2010) used nanofibrous carriers-mediated siRNA delivery to enhance neuronal differentiation of stem cells. By using a mussel- inspired coating with strong adhesive properties, siRNA complexed with cationic transfection reagent (Lipofectamine RNAimax) was immobilized on the electrospun PCL nanofibrous scaffolds. Application of this system resulted in significant enhancement of neuronal differentiation of mouse neural progenitor cells due to the synergistic effects of 3D nanofibrous topographical cues and carriers-mediated knockdown of the REST [130].

## CONCLUSION

In the treatment of HD, so far there is a limited success rate in modifying disease symptoms by traditional pharmacological agents. Nowadays advances in drug delivery and cell/ tissue engineering open the possibilities to achieve, beyond symptomatic relief in HD, neuroprotection and neurorepair. RNAi engineered nanotherapeutics has emerged during these years as an innovative strategy to control stem cell fate *in vitro* and *in vivo*. Nano and microcarriers for nucleotide delivery offer numerous benefits over lipoplex transfection, electroporation and lentiviral transduction. Nano and microcarriers based delivery of cells, growth factors and RNAi may be among the best means for controlling the differentiation and survival of the delivered cells while stimulating neuroprotection and repair.

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# CHAPTER I

## INTRODUCTION

*In vitro* cell culture models are of particular importance in Neuroscience Research. While single cell cultures are widely used, the organotypic brain slice culture model is the closest to an *in vivo* situation. Indeed, brain slices contain all the cells present in the brain and maintain its three dimensional architecture [39]. Brain slice cultures allow maintaining the survival of all cell types, the functionality of synapses and neuronal properties. In other words, the basic cellular and connective organization of the donor brain regions are well preserved, thus the slice cultures provide an easily accessible platform to study: neurodegeneration, mechanisms of disease (Alzheimer, Parkinson's disease, Huntington's disease), and test different treatments such as pharmacological treatments, cells replacement or tissue engineering treatments [42,124].

The first description of organotypic brain slices have been described for the culture of dorsal cerebellum in 1970 [125,126]. Then the culture of brain slice model was perfectly developed in 1982 using spinal cord-dorsal root ganglia [127]. The characterization and the improvement of this unique *ex vivo* model was realized by Gähwiler and Hefti (1984) with roller tube cultures [128]. The technique was modified as a permanent culture model on membrane inserts [129,130] and is nowadays used by several research groups [131–133]. Slices can be cultured as single slices from a respective brain area or as a co-culture where two functional related brain slices are connected. Since then *ex vivo* models of Parkinson's disease, Alzheimer, cerebral ischemia, HD have been developed (For a review see [42])

In the case of HD, organotypic model development dates back to 1986 with the study of the effects of KA administration on glial cells, both in the animal model of HD (which uses this drug to damage GABAergic neurons) and in striatal organotypic cultures [44]. Since then other genetic and non-genetic models have been developed to model HD.

Genetic models of HD derive principally from animal models expressing a truncated [23] or full length [24,25] form of mutant htt (mhtt). This model mimics the pathology including the genetic aspect of HD and as a consequence

leads to the MSN death. The model acquirement does not demand specific knowledge but may be more expensive in a long term as genetic mice need to be purchased. The R6/2 and knock-in Q175 mouse are mostly used, from mice of 12 weeks old until 1 year or more [134–136]. So usually, organotypic slices deriving from genetic models are made with adult brain. These brain slices are more difficult to prepare as the cranium is unbending and the culture of adult brain is more complicated. Indeed, most brain slice culture systems have used the perinatal rodent brain, taking advantages of its higher viability relative to the older one. The survival of adult brain slices did not exceed 1 week in cultures [137] with serum and 3-4 weeks without serum [138].

Another way to obtain genetic models of HD brain slices is the transfection of HD-polyQ-GFP plasmids directly onto the slice. Three different non-viral transfection methods were tested on mouse cerebellum organotypic slices [139]. And then authors proposed an innovative model obtained in 72h with a transfected mutant huntingtin gene which when exposed to malonate produced HD-like lesions and provided a new model of HD, conserving the correlation between CAG repetitions and aggregation length [140]. This new model is powerful because in 72h they obtained a genetic model of HD, but even with the best transfection methods the maximum of cells containing the plasmid were around 35%. Slice transfection requires high technology equipments and a skilled operator, which make them difficult to use in routine.

Non genetic *ex vivo* models of HD have been performed using neurotoxins (KA [44], IA, QA [48], NMDA [52], 3-NPA [46]) added into the media, which then mainly affect the striatum, the cortex and the hippocampus, inducing neuronal loss [36,49,141]. Striatal degeneration of HD can also be caused *in vivo* by 3-NPA, a mitochondrial inhibitor. This model allows understanding the neurotoxicity and the excitotoxicity mechanisms [46]. Although only the cellular aspect of HD can be studied, they present many advantages: they are easy and rapid to develop and do not involve high economic impact as normal rodents are used. For these models, usually newborn or one day old rodent are sacrificed and cultured during 3-4 weeks and then brain slice cultures are exposed for 48h to neurotoxins. In this way, a model is obtained in 1 month,

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which is quite some time. Moreover, the heterogeneity of the results after treatment evaluation prompts to focus on another system.

In this work, we propose a novel and simple approach to model HD. We developed a coronal organotypic culture model obtained just by vibrosectioning of the brain. This brain slice model allows selecting the area that we want to study. The main areas involved in HD are obtained in a unique slice without neurotoxins to induce the GABAergic MSNs depopulation. The aim was to induce progressive striatal MSNs degeneration in a single step while preparing the slices, in order to obtain a simple reproducible HD *ex vivo* model. To our knowledge, this is the first time that vibrosection was investigated for modeling HD. We have already demonstrated the interest of vibrosectioning the brain for developing a fully characterized *ex vivo* PD model. Interestingly, we obtained all the areas involved in PD: cortex, striatum, ventral pallidum the visualization of the fibers present in the medial forebrain bundle and the substantia nigra[133]. Based on the same method, we elaborated this unique model of HD.

*Publication in process for Neuroscience methods*

# **PUBLICATION N°1**

**A METHOD FOR A SIMPLE IN VITRO**  
**HUNTINGTON'S MODEL: SLICE CULTURE FOR**  
**MODELING THE DISEASE AND STUDY THE REPAIR OF**  
**THE GABAERGIC NEURONS**

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## ABSTRACT:

**BACKGROUND:** Organotypic brain slice cultures represent an excellent compromise between single cell cultures and animal. They preserve brain three-dimensional architecture, synaptic connectivity and brain cells microenvironment. This model has allowed researchers to observe cellular interactions and mechanisms through a simple and rapid method. Moreover, slice culture model systems provide a unique opportunity to monitor the circuits' repair in a dish after cells transplantation without any concern about the possible interaction between neurotoxins and cells grafted.

**NEW METHODS:** We aim to develop a novel model of Huntington's disease. We have generated a coronal slice from rats' brains that includes all the areas involved in HD in a single slice preparation, without using neurotoxins to induce the lesion.

**RESULTS:** After investigated different axis to cut the rats' brains, we determined that coronal brain slices can be cultured during approximately 3 weeks with a preservation of normal cyto-architecture except in the striatum. After 1 week, we observed a reduced volume of the striatum associated with the decrease of GABAergic medium spiny neurons markers mimicking HD cellular hallmarks.

**COMPARISON WITH EXISTING METHODS:** This organotypic model involves GABAergic MSNs degeneration simply by cutting the tissue with a specific axis. Inversely, the use of neurotoxins such as quinoleic acid to model Huntington's disease lead to heterogeneity in the results obtained, which must be taken into consideration.

**CONCLUSIONS:** This unique model presents a new approach for modeling Huntington's disease *in vitro*, and provides a useful innovative method for screening new potential therapies for neurodegenerative diseases.

## Keywords:

Organotypic slices, Huntington's model, neurodegenerescence, methodology, DARPP32, GAD67

## Abbreviations:

DARPP32: Dopamine- and cAMP-Regulated neuronal PhosphoProtein of 32kDa; GAD67: Glutamate decarboxylase 67; GSBS: Grey's Salt Balanced Solution; HD: Huntington's disease; HTT: Huntington gene; htt: protein huntingtin; MEM: minimum essential medium eagle, MSN: medium spiny neurons; NeuN: neuronal nuclei; PBS: phosphate buffered saline; PFA: paraformaldehyde.

## 1. INTRODUCTION

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder with a general prevalence of about 10 per 100.000 births [1,2]. This high CAG triplet repetition is localized in the IT-15 gene of the chromosome 4: the huntingtin gene (HTT). This repetition will lead, at the protein level, to a polyglutamine repetition at the NH<sub>2</sub>-terminal part of the huntingtin protein [3]. The accumulation of the mutant htt protein is excitotoxic, therefore it progressively compromises survival and normal neuronal functioning, primarily in the striatum or caudate/putamen and progresses with the degeneration of cortical pyramidal neurons, mainly projecting to the striatum [4]. Striatal GABAergic medium spiny neurons (MSN), which constitute the majority of cells in this region are affected by this pathology. They extend axonal projections to the external segment of the globus pallidus (GP), and to the substantia nigra *pars reticulata*. The progressive loss of these neurons is accompanied by a corresponding ventricular enlargement and gliosis [5]. Clinically, this neurodegenerative disorder leads to involuntary movements, cognitive impairment and psychiatric manifestations [6]. Medications are available to help managing the symptoms of Huntington's disease, but treatments can't prevent the physical, mental and behavioral progressive decline associated with the disease.

Novel drug and cell therapy approaches in development require extensive evaluation before use in humans [7]. Considering the role of mHtt in the pathogenesis of HD, this protein has been transfected in different cell lines for the study of HD *in vitro*. [8] Recent studies have explored the use of embryonic stem cells and induced pluripotent stem cells as cellular models for disease research and the development of biomedical applications. Significant work being done in the field is the establishment of human iPSC lines from patients with HD mutations [9]. Although these models are relevant to study important hallmarks of HD, allowing the investigation of key intracellular mechanism involved in the disease, as well as the identification of novel pharmacological targets, *in vitro* models do not reflect the complexity of the disease. The effect of the microenvironment, and the functional aspects of the disease can only be

provided by *in vivo* models. These models can be broadly divided into genetic models presenting the mutated htt gene in various forms or into neurotoxic models showing a quite specific GABAergic MSN degeneration [10]. These models have been designed to elucidate the pathogenesis, cell death mechanisms and to evaluate therapeutic strategies for HD [11]. But *in vivo* studies require high technical and financial resources and they do not allow to simultaneously test several conditions in the same animal [12–14]. For innovative cell therapy or tissue engineering studies it is very complicated and time consuming to understand and elucidate all the interactions between grafted cells and host brain. Even more, generally human cells are grafted into rats or mice models involving an immunological component that needs to be taken into account in these models.

Organotypic brain slices, which can be maintained in culture for several weeks, are commonly used in brain disease research as they provide unique advantages over *in vivo* and *in vitro* platforms [15,16]. They largely preserve tissue structures, maintain neuronal activities and synapse circuitry, and replicate many aspects of the *in vivo* context [17]. Further advantages of these brain cultures reside in their low-cost, rapid preparation and the use of many brain slices/animal allowing the study of an important number of conditions. Thus, gene functions and pathways can be easily studied as in an *in vitro* system.

Recently, organotypic brain slice cultures have been used for HD modeling. Two major techniques can lead to this model. The first one uses organotypic slices made directly from transgenic mice expressing HD patterns, such as R6/2 transgenic mice [18,19]. But these models require the use of adult tissue, as mice develop the disease over time. This adult tissue is very difficult to manipulate in *ex vivo* conditions, the cranium is unbending and the organotypic slices are not viable over time. The survival of adult brain slices did not exceed 1 week in culture with serum [20] and around 3 weeks without serum [21]. Indeed, most brain slice culture systems have used the perinatal rodent brain, taking advantages of its higher viability relative to the older one. In the second technique, organotypic slices were prepared from normal rodents and GABAergic neuron loss was obtained by injecting neurotoxic agents such as

quinolic acid or 3-NPA, a mitochondrial inhibitor, added in the media. However, the use of neurotoxins leads to heterogeneity in the results, which must be taken into consideration. Furthermore only the cellular aspect of HD can be studied, in detriment of the genetic component of the pathology [22–24]. Another solution is to transfect the slices with HD-polyQ plasmids or with DNA constructs derived from the human pathological HTT gene [25–27]. However, slice transfection involves high technology equipment and a skilled operator as organotypic brain slices are delicate and frequently become damaged during the preparative stages [16]. Notwithstanding these obstacles, the introduced exogenous proteins into both neuronal cells have been consistently difficult to achieve.

In this study, we developed a novel approach to model HD. We developed a coronal organotypic culture model that includes the main areas involved in HD in a unique slice that does not need neurotoxins to induce the GABAergic MSN depopulation. The aim was to induce progressive striatal MSNs degeneration in a single step while preparing the slices, in order to obtain a simple reproducible HD *ex vivo* model. We first studied and characterized different axis to cut the whole brain to determine the one that induces the degeneration of MSN. Then we explored the viability of this unique slice containing all the areas affected by the disease and characterized the decrease of striatal GABAergic neurons visualized with DARPP32 and GAD67. Our study is the first one describing a cheap, simple and reproducible model of HD without neurotoxin.

## **2. MATERIALS AND METHODS**

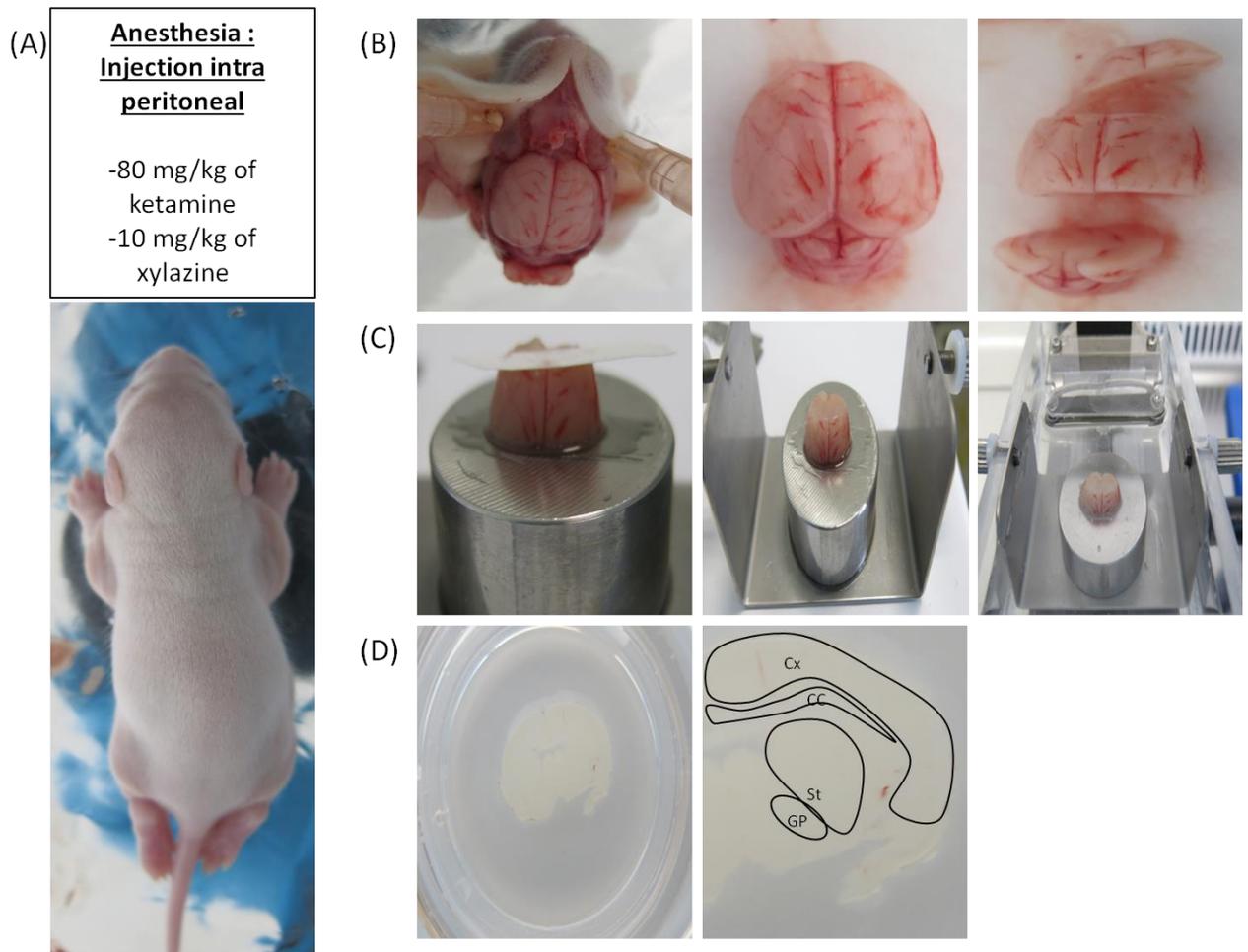
### **2.1 PREPARATION OF ORGANOTYPIC SLIDES**

Animal care and use were in strict accordance with the regulations of the French ministry of agriculture and all animal procedures were approved by the animal experimentation ethic committee of “Pays de la Loire”. Every effort was made to minimize animal suffering and the number of animals used.

Timed pregnant Sprague-Dawley rats were purchased from Janvier (St Berthevin, France), or from SCAHU (Service commun d'animalerie hospitalo-universitaire, University of Angers, France). Postnatal 6 to 8 days old pups were

used to prepare organotypic slices according to the Stoppini method [28] recently modified [29] by our team. Before starting the experiment, solution 1 (**Table 1**) needs to be prepared and kept at 4°C and the membrane has to be hydrated with solution 1 for at least 30 min. Animals were rapidly sacrificed after intraperitoneal injection of 80 mg/kg of ketamine (Clorketam 1000, Vetoquirol, Lure, France) and 10 mg/kg of xylazine (Rompum 2 %, Bayer Health Care, Kiel Germany) (**Figure 1A**). Brain are removed and rapidly dissected (**Figure 1B**) before being glued onto the chuck of a water-cooled vibratome. This gesture must be quick in order to preserve the brain, and immediately immersed in the buffer solution (**Table 1**) at 4°C (**Figure 1C**). Under aseptic conditions, 400 µm slices were cut in different configurations in order to obtain a progressive degeneration of the GABAergic MSNs. Finally, cerebellum and olfactory bulbs/prefrontal cortex were cut off and brains were glued, onto the chuck of a water cooled vibratome (Motorized Advance Vibroslice MA752, Campdem instruments). Coronal sections were collected and placed in sterile ice-cold Grey's Salt Balanced Solution (GSBS) (Sigma Aldrich, St Louis, USA) supplemented with 6.5 mg/L of glucose and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B) (Sigma Aldrich, St Louis, USA) for one hour (**Table 1**).

Slices were cultured in two different media. From days 0 to 3, a serum containing medium was used: 50 % MEM (Minimum Essential Medium Eagle, Sigma Aldrich), 25 % Hank's (Hank's Balanced Salt Solution, Sigma Aldrich), 25 % of horse serum (decomplemented horse serum, Gibco), 6.5 mg/mL of glucose, 1 mM of L-glutamine (Sigma Aldrich, St-Louis, USA) and 1 % of antibiotics (Sigma Aldrich, St-Louis, USA) (**Table 2**). From days 3 to 18, a serum free medium was used: Neurobasal medium (Gibco, Life Technologies, Paisley, UK) supplemented with 6.5 mg/L of glucose, 1 mM of L-glutamine, 1X B27 supplements (Gibco, Life Technologies, Paisley, UK) and 1% of antibiotics (**Table 2**). The media was changed the first day after of culture and was then renewed every two days during the entire culture period.



**Figure 1. Protocol to obtain organotypic slices.** Rats' anesthesia must be realized with an intraperitoneal injection of ketamine and xylazine, in a solution of NaCl 0,9 %. (A) The head must be dissected quickly and carefully to preserve brain structure. Cortex and cerebellum should be cut (B). Brain is glued onto the chuck of a water-cooled vibratome and plunged in a cold solution containing high level of glucose. Under aseptic conditions, 400 $\mu$ m thick whole brain sections are cut and collected (C) The brain slices are disposed in a 0.4  $\mu$ m membrane insert with media 1 solution and incubate in 5% CO<sub>2</sub> and observation of the coronal slice. Cortex (Cx), striatum (St), globus pallidus (GP) and Corpus Callosum (CC) can be easily observed (D).

Typically, about 10 slices can be obtained per brain. The first two and the last two brain slices did not contain the main areas involved in the pathology and were discarded. Four slices per animals were next transferred to 30 mm diameter semiporous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and incubated at 37°C and 5 % of CO<sub>2</sub>. A total of about 20 rat pups and about 80 organotypic slices were necessary to perform the whole characterization. For each condition, a minimum of three slices taken from three different rat pups were used.

Products	Quantity	Special instructions
Gey's balanced salt solution	50 mL	Must be at 4°C
D-glucose	6.5 mg/mL	Must be filtrated at 0.22 $\mu$ m
Antibiotics	1 %	Streptomycine and penicilline

**Table 2.** Description of the buffer solution needed during the first step of organotypic slice preparation. This solution must be prepared under aseptic conditions.

Solution	Products	Quantity	Special instructions
<u>Media 1</u>  (From day 0 to day 3)	Minimum Essential Medium Eagle (MEM) Alpha Modifications	50 %	sterile
	Hanks balance salt solution	25 %	sterile
	Horse serum	25 %	Must be inactivated
	D-glucose	6.5 mg/mL	Must be filtrated at 0.22 $\mu$ m
	L-glutamine	1 mM	Must be filtrated at 0.22 $\mu$ m
	Antibiotics	1 %	Streptomycine and penicilline
<u>Media 2</u>  (From day 3 to the end of the experiment)	Neurobasal media		sterile
	D glucose	3.25 mg/mL	Must be filtrated at 0.22 $\mu$ m
	L-glutamine	1 mM	Must be filtrated at 0.22 $\mu$ m
	B27	2 X	
	Antibiotics	1 %	Streptomycine and penicilline

**Table 1.** Description of the media needed during all the organotypic slices culture. These media should be prepared under aseptic conditions. Media should be renewed every 2 or 3 days during culture.

## 2.2 SLICE ANGLE SELECTION TO OBTAIN A HUNTINGTON'S DISEASE MODEL.

Brains were cut with different angles to obtain the most complete DARPP32 and GAD67-positive cell degeneration over time. For this analysis,

three angles have been chosen: sagittal, coronal and transversal. For sagittal slices, brain hemispheres were separated and glued onto the chuck of a water-cooled vibratome and slices were cut alongside of the midline. Concerning coronal slices, cerebellum and olfactive bulbs/prefrontal cortex were cut off and brains were glued, on their dorsal side, onto the vibratome chuck. At last, to perform transversal slices, the underside of the brain was glued on the vibratome chuck. For each condition, 400  $\mu\text{m}$  slices were performed with razor blade angle of  $14^\circ$ .

## 2.3 HISTOLOGICAL STUDIES

At different times, ranging from 0 to 19 days, organotypic slices were washed with phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium), fixed with 4 % paraformaldehyde (PFA) (Sigma–Aldrich, St Louis, MO, USA) in PBS pH 7.4 for 2 h and then washed three times with PBS. Finally, slices were removed from membrane inserts and stored at  $4^\circ\text{C}$  in PBS until use.

### 2.3.1 DARPP32 and GAD 67 Immunohistochemistry

A mouse anti GAD67 antibody (5  $\mu\text{g}/\text{mL}$ , clone 1G10.2, Millipore SA, Guyancourt, France) and mouse anti-DARPP32 (250  $\mu\text{g}/\text{mL}$ , clone 15, DB science, Le Pont de Claix, France) were used to observe striatal-globus pallidus GABAergic pathway. Slices were incubated 48 h with the primary antibody diluted into PBS, BSA 4 % at  $4^\circ\text{C}$ . After washes, slices were incubated with the biotinylated anti-mouse secondary antibody (7.5  $\mu\text{g}/\text{mL}$ , Vector Laboratories, Burlingame, USA). Then slices were washed and quenching of peroxidase was performed with 0.3 %  $\text{H}_2\text{O}_2$  (Sigma, St Louis, USA) in PBS, at RT for 20 min. After PBS washes slices were incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) in PBS at RT for 1 h. Sections were then washed and revealed with 0.03 %  $\text{H}_2\text{O}_2$ , 0.4 mg/mL diaminobenzidine (DAB, Sigma, St Louis, USA) in PBS, 2.5 % nickel chloride (Sigma, St Louis, USA) and dehydrated before mounting.

### 2.3.2 Quantification of DARPP32 and GAD 67 positive fibers and cells

GAD67-positive fibers and DARPP32-positive neurons were quantified in the striatum at different time-points, from 0 until 19 days post-lesion, using the

Metamorph® software from Molecular Devices. Results were presented as mean differences +/- average deviation and were calculated from 6 independent pictures taken from 4 different rats for each group.

### 2.3.3 [NeuN Immunohistochemistry](#)

An antibody against neuronal nuclei (NeuN) (clone A60, Merck Millipore, Billerica, MA, USA) was used to observe the viability of cells within the brain organotypic slices. Slices were incubated 48 h with the primary antibody diluted into PBS BSA 4 % (1/50) at 4°C. After washes, slices were incubated with the biotinylated anti-mouse secondary antibody (7.5 µg/mL, Vector Laboratories, Burlingame, USA). Then slices were washed and quenching of peroxidase was made with 0.3 % H<sub>2</sub>O<sub>2</sub> (Sigma, St Louis, USA) in PBS-T, at RT for 20 min. After PBS washes, incubation with Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) in PBS was performed at RT for 2 h. Sections were washed and revealed with 0.03 % H<sub>2</sub>O<sub>2</sub>, 0.4 mg/mL diaminobenzidine (DAB, Sigma, St Louis, USA) in PBS, 2.5 % nickel chloride (Sigma, St Louis, USA) and dehydrated before mounting.

### 2.3.4 [Quantification of NeuN-positive neurons](#)

The survival of total neurons in certain organotypic slice regions was estimated by immunohistochemistry against NeuN. NeuN-positive neuron was calculated, from days 0 to 19 using the Metamorph® software. At each time-point, six pictures taken from three different slices showing cortex, striatum and lateral septum were used. NeuN-positive neuron in these areas was expressed as a percentage of NeuN staining at day 0 considered as positive control. Results were presented as mean differences ± average deviation.

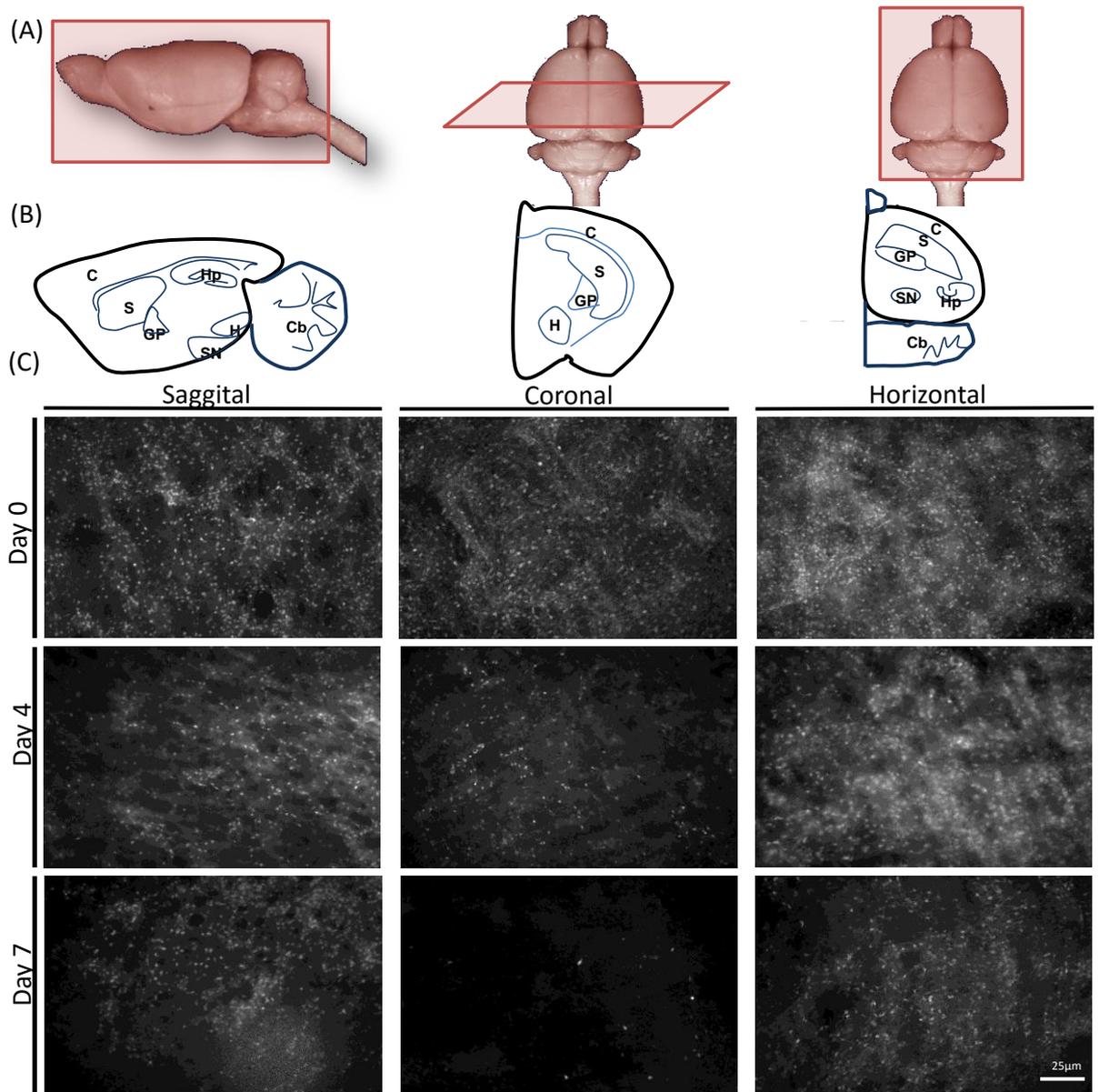
### 2.3.5 [Statistical analysis](#)

Data are presented as the mean value of three independent experiments ± standard deviation (SD), unless otherwise stated. Significant differences between samples were determined using an analysis of variance (ANOVA) test, followed by a Scheffe post hoc test which indicates if conditions were significantly different. *P*-value was set to 0.05, unless otherwise stated.

### **3. RESULTS**

#### **3.1 SLICE ANGLE SELECTION TO OBTAIN A HUNTINGTON'S DISEASE MODEL.**

To determine the best model of GABAergic MSN neurodegeneration, mimicking one of the pathological hallmarks of HD, the neonatal brain was cut following three different axes to obtain the organotypic slices (**Figure 2A and 2B**). With the three types of sectioning planes the striatum, cortex and globus-pallidus, the brain areas mainly affected in HD were visible. The progressive degeneration of MSN was evaluated by DARPP32 expression. We observed that DARPP32 staining was present and observable in the whole striatum independently of the slice plane chosen (**Figure 2C**). Moreover, in horizontal and sagittal slices, DARPP32 positive cells were still highly present in the striatum after 4 days and even after 7 days of culture (**Figure 2C**). In coronal sections (**Figure 2C**), the number of DARPP32-positive cells was reduced by around 30% after 4 days and up to 70% after 7 days. As a quick degeneration of DARPP32-positive cells was desired, coronal sections were used for the rest of this study. Moreover, the sections can be kept as a whole therefore containing the striatum from the two hemispheres, providing a control in the same slice. If desired, and in order to increase the viability of the slices they can also be cut along the midline.



**Figure 2. Slice angle selection to model HD.** Rat's brain was cut in order to obtain sagittal, coronal or horizontal brain slices (A). Which each different angles, we can visualize all the areas involved in the HD (B) DARPP32 was detected in brain slices obtained by immunofluorescence at day 1, 4 and 7 after sagittal, coronal and horizontal (C) sections.

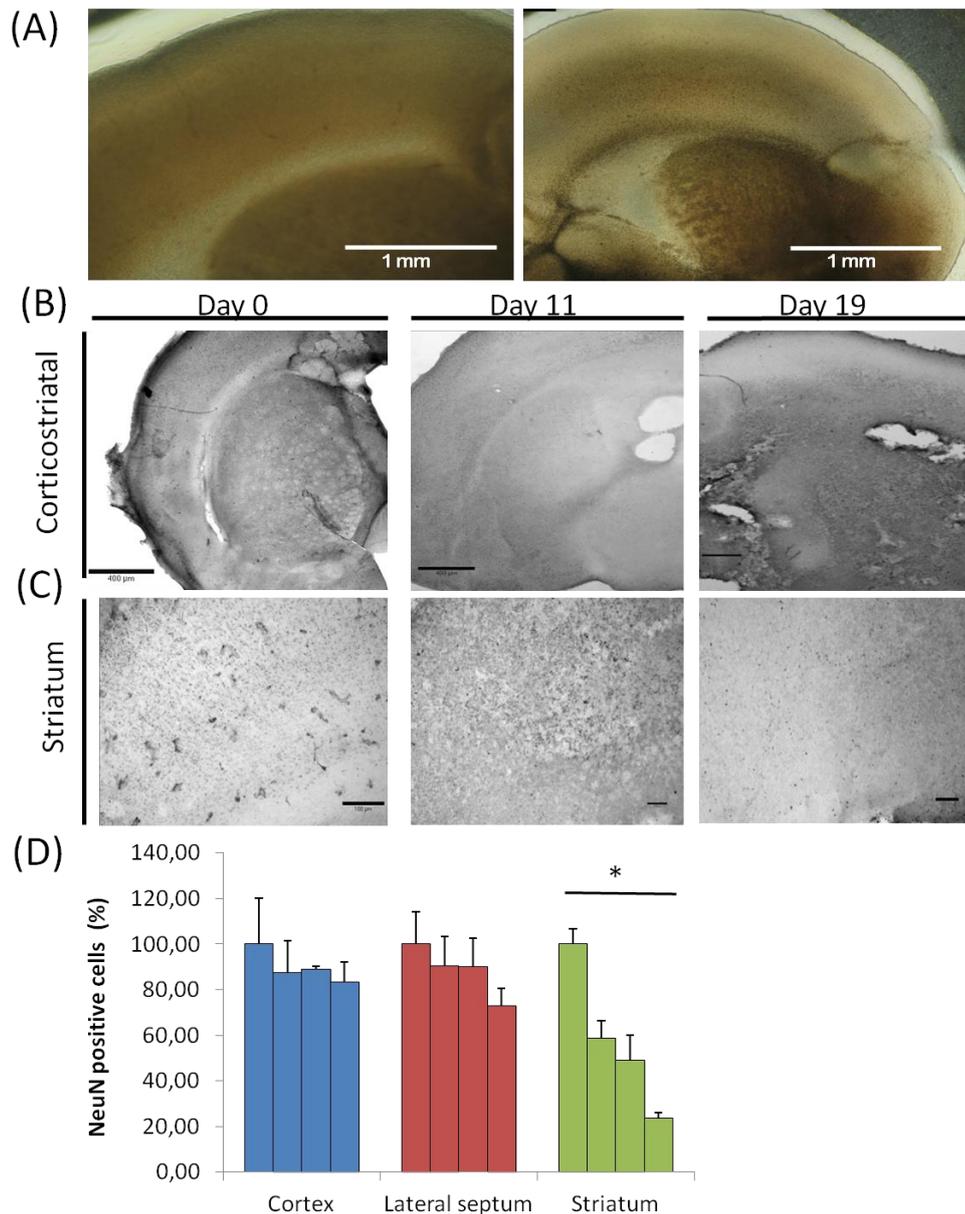
### 3.2 ORGANOTYPIC SLICES MORPHOLOGY AND VIABILITY OF CULTURED NEONATAL BRAIN SLICES.

Culture conditions remarkably affect the organotypic culture viability. We already described a protocol using 2 specific media with and without serum [29]. Using these conditions, the whole coronal HD organotypic slices can be kept viable during at least 3 weeks in culture (**Figure 3**). We observed a decrease of striatum volume from 4 days onwards and a good conservation of organotypic

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slice's morphology during 3 weeks (**Figure 3A**). No important distortion or flattening of the slices was detected. However, an important thinning of the striatum within 6 days was observed by bright field microscopy, predicting a reduction of viability of the striatal neurons (**Figure 3A**).

To confirm the viability of organotypic brain slices, neurons were visualized with an antibody against NeuN and quantified at day 0, 7, 11 and 19. During the 3 weeks of culture the number of NeuN-positive cells decreased less than 20 % +/- 4 % in the cortex and lateral septum compared to striatum, (**Figure 3C and 3D**), underscoring the viability of the neuronal cells in these areas. However, only 20 % +/- 5 % of NeuN-positive cells were counted in the striatum, explaining the decrease of volume observed during the culture and confirming the neurodegeneration (**Figure 3**).

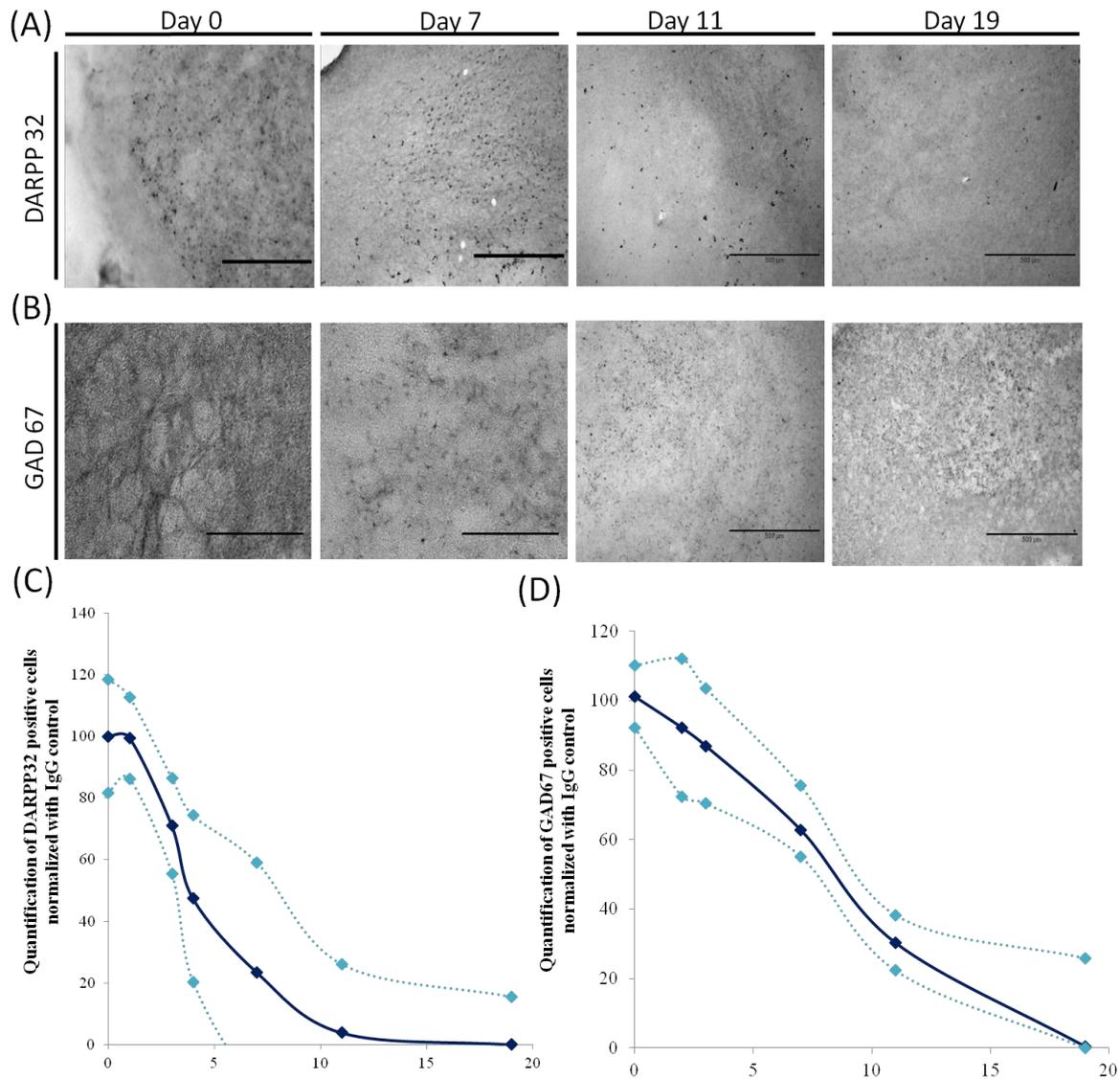


**Figure 3. Morphology and viability of organotypic slices.** Observation of coronal brain slices was realized by bright field microscopy (A). Immunohistochemistry against neuronal nuclei (NeuN) showing three brain regions: cortex, striatum and lateral septum at day 0, 11 and at day 19 (B) at the bottom. Immunohistochemistry against neuronal nuclei (NeuN) showing the striatum (C) Quantification of NeuN-positive cells in cortex, striatum and SN shows no important loss of staining in the cortex after 19 days in culture (D). While NeuN expression decreased by 80 % and 27 % in the striatum. \*: Significantly different results with  $n = 3$  and  $P$ -value = 0.05. Pictures about organotypic slides until day 19.

### 3.3 ORGANOTYPIC SLIDES CULTURES DISPLAY PROGRESSIVE DEGENERATION OF GABAERGIC PATHWAY

In order to demonstrate the degeneration of medium spiny neurons in the striatum, we followed by immunohistochemistry the progressive decrease of DARPP32 (**Figure 4A**) and GAD67 (**Figure 4B**), which are markers of

GABAergic MSNs. These markers were perfectly specific because we didn't find any positive cells in the cortex (**Figure 4A and 4B**). The number of DARPP32-positive neurons and GAD67-positive neurons decreased over time with around a 50% decrease during the first week when compared to day 0 (**Figure 4A and 4B**). A quantification of the GABAergic marker density GAD67 shows a progressive loss of 30 % at day 4, 45 % at day 7 and 70 % at day 11 when compared to day 0. It was almost complete by day 19 with an average of only 1 % staining left. However, the staining intensity varied between slices due to the tissue damage (**Figure 4D**). Indeed, a more dramatic loss of DARPP32 staining was detected over time with an average of 25 % decrease at day 2, 50 % at day 4 and 75% at day 7 compared to day 0, and was complete by day 19 (**Figure 4C**). Even though the average loss of DARPP32 staining was a bit more rapid the profile of these 2 graphs are similar, confirming the progressive loss of GABAergic neurons in the organotypic slices.



**Figure 4. Modelisation of specific MSN markers.** Immunohistochemistry against striatal A) DARPP32 positive neurons and B) GAD67 positive neurons at day 0, 7, 11 and 19. C) Striatal DARPP32 positive neurons decreased progressively until day 11 and became total at day 19 in comparison with day 0 which represents 100% represented with SD in doped lines. D) Striatal GAD67 positive neurons quantification with SD in doped lines. A 10% decreased was detected at day 2 which reached 30 % at day 4, 45 % at day 7 and reached 100 % at day 19, holding the fact that day 0 represent 100 %. N=4

## 4. DISCUSSION

Organotypic slices have been widely used as model of neurological pathologies including Parkinson's disease, brain stroke and cerebral ischemia [16]. In the present work an innovative *ex vivo* model of HD in which progressive striatal MSN degeneration was obtained in a single step while preparing the slices, was developed. The MSN degeneration occurred in around

three weeks and was specific to the striatum as the viability of the other brain regions was maintained during this time. We didn't observe morphological modification except for the volume reduction in the striatum confirming what is observed in HD. This simple model thus allows evaluating different neuroprotective and neuroreparative therapeutic strategies. Organotypic slices were obtained from 6 to 8 day old pups. It is well known and established that tissue from embryonic or post natal donors survive better [30,31]. Slices post-natal donors is recommended because at this age, brain slices will conserve a better morphology, an increase survival and we will obtain a more stable models regarding to the lesions. Animals were sacrificed between days 6 to 8 which provides a perfect time window for establishing brain slice cultures on membranes. More particularly, for Parkinson's disease the slices are principally harvested between 4 and 9 weeks after birth [32,33].

Hypothermia has been shown to improve the preservation of hippocampus brain slices [34–36]. In our study, in order to preserve brain slices we insured that the brain was kept at 4°C in solution buffer containing high percentage of glucose during the whole slice preparation process. We chose not to place the head in ethanol 70 % bath as some teams have reported [31] in order to maintain physiological conditions of organotypic brain slices. Within the same line, the media containing horse serum was only used during the first 3 days even though horse serum diminishes tissue flattening, and promotes survival of neurons, astroglia or microglia in organotypic brain slices [37]. In order to diminish the variability between slices due to changes in serum batches we then used a serum-free media containing glucose, glutamine, antibiotics and B-27<sup>®</sup>. Indeed, serum increased the degeneration of neurons in brain slices when the media contain serum for a long-time period [21]. In a previous study, we confirmed the beneficence of using horse serum during a short period followed by “synthetic” media and according to these results, we applied this method to culture organotypic brain slices modeling HD. In this way, we are able to maintain the viability of brain *ex vivo* for at least 3 weeks, and obtained the HD model in 5 days. Other studies demonstrated the benefit to culture organotypic slices without serum such as our study, and brain slices from transgenic mouse or rat brain slice with injection of neurotoxins were principally cultured with

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artificial cerebrospinal fluid or specific media containing HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [38–40].

This organotypic model involves GABAergic MSNs degeneration due to mechanical cutting of tissue between the striatum and substantia nigra, which leads to retrograde and anterograde neuronal degeneration in the substantia nigra and in the striatum, respectively. In this report, a complete characterization of the behavior of MSNs and GABAergic pathway was assessed by immunostaining through the visualization of the decrease of DARPP32 and GAD67, two specific markers of MSNs [5]. It revealed that a rapid loss of DARPP32-positive neurons occurred in the striatum followed by a decrease in GAD67 staining. A decrease of 50% and 30% of the DARPP32 and the GAD67 positive cells respectively was detected 4 days after slice preparation compared to day 0. This allows the development of an early model of the disease, in which a window of time of at least 15 days was obtained to study new therapeutic approaches. One important reason to culture brain slices is to study and characterize the interaction between neuronal populations. The coronal section allows studying those parameters, thanks to the observation of the globus pallidus, the striatum and all the structures involved in the HD in one slice. We can easily imagine testing different molecules with therapeutic effect such as growth factors directly onto slices or in the media and observe their effect in all the MSNs afferents and projections [41,42]. [43]. Indeed, with the same method, we developed an innovative model of PD and we were able to evaluate new therapeutic approaches combining stem cells and biomaterials releasing growth factors in order to treat the disease or to reduce the related symptoms. [29]

In summary, this model represents a promising tool to quickly and efficiently test innovating treatment in the HD such as regenerative medicine with stem cells and biomaterials. The development of simple and non expensive model with precise characterization of neurodegeneration could allow to easily study the therapeutic effect of treatment but also the role of microenvironment in the response of the treatment.

## CONCLUSION

In this study we developed and characterized a relevant *ex vivo* model of HD using membrane techniques culture and specific media. This new model can be use to study the early stage of the pathology because we observed the reduction of striatum such as in the HD in 4 days. This represents a perfect model to study the relevance of new and innovative treatment.

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## DISCUSSION

The use of organotypic slices allowed the development of a new simple model of Huntington's disease. Indeed, GABAergic MSNs degeneration is observed after mechanical cutting necessary to obtain brain slices. In this way, no other supplementation with neurotoxins, quinoleic acid or 3-NPA is needed for the progressive loss of striatal neurons, which reduces the risk of heterogeneous results. Usually, only some areas such as the striatum, the hippocampus or cortex and striatum are used in *ex vivo* models of HD[42] [44,52] Here we have all the regions involved in HD, the cortico-striatal-globus pallidus area of one hemisphere or of both hemispheres allowing to use one side as control.

Nonetheless, the full characterization of this model is required. Indeed, we followed NeuN and GABAergic markers for only 19 days. We should know how much time brain slices survive and understand exactly the mechanism of the decrease of DARPP32 and GAD67. DARPP-32 is a fundamental component of the dopamine-signaling cascade, and its expression is essential to the ability of dopamine to regulate the physiology of striatal neurons. In our study, the decrease of GAD67 is progressive and comparable to NeuN while DARPP32 decreased drastically from day 5. The results presented here suggest a selective impairment in several aspects of dopaminergic signaling. Indeed, dopaminergic signaling has been already described in the literature to be involved in presymptomatic defects and contribute to HD pathology [142], and DARPP32 is the first marker decreasing. DARPP32 is also a mediator of signaling cascades in dopaminergic neurons which corroborate this hypothesis. So, our model seems to be severely impaired in GABAergic neurons' ability to regulate the physiological state of striatal neurons via dopamine. To confirm our hypothesis, we can imagine checking the dopamine-regulated ion channels by electrophysiological studies and determine if a reduction in the striatal-specific dopamine targets, PKA, DARPP-32, ARPP-16, and ARPP-21 is associated, with RT-qPCR or immunofluorescence.

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The aim of this study has been to develop a simple model to screen potential treatment including cell replacement with MIAMI cells combined with PAMs. This model allows the development of an early model of the disease, in which a window of time of at least 7 days was obtained if we considered DARPP32 and 14 days if we considered GAD67. The timing differences are evidenced when comparing this model with those in which organotypic brain slices are cultured during 3 weeks followed by the addition of neurotoxins in the media in order to mimic HD [44–47]. Thus, stem cell transplants coupled or not with MPA in this model enable the evaluation of the survival and differentiation of grafted cells and the study of the behavior of the host tissue in particular related to the evaluation of the secretion of neurotrophic factors by stem cells that can be made during 14 days. We can also easily imagine testing different molecules with therapeutic effect such as growth factors directly onto slices or in the media and observe their effect in all the MSNs afferents and projections [143,144]. In summarize, our model is so much faster, simpler and less expensive than the *in vivo* model.

# CHAPTER II

## INTRODUCTION

Transplantation of stem cells for the treatment of HD garnered much attention prior to the turn of the century. Several studies using MSCs have indicated that these cells have enormous therapeutic potential in this disease [145,146]. In the case of HD, the beneficial effect of stem cell transplantation should be provided by the generation of new graft-derived neurons and by the secretome of these cells [147]. MSCs are not only able to differentiate into neuron-like cells but they also exert paracrine effects by modulating the plasticity of damaged host tissues.

The trans-differentiation of MSCs into neural lineage has been achieved *in vitro* by culturing them with specific media. This media is often composed of growth factors or small molecules involved in neuronal lineage differentiation. The degree to which the cells will differentiate varies depending on the protocol and cells used [148,79,149]. In all cases, although the MSCs were committed to neuronal lineage, the functionality at the end of the process was still lacking. Attempting to understand how to improve the differentiation, REST also known as neural-restrictive silencing factor (NRSF) has been proposed as it functions as a master negative regulator of neurogenesis [150]. REST is a zinc finger transcriptional repressor able to bound the RE-1 sequence in his target gene promoter which allows recruiting histone deacetylases and methyltransferase to modulate the chromatin structure [151,152]. In non-neural cells, REST is abundant and represses neuronal genes. Importantly, REST itself is differentially regulated throughout neural development. In MSCs, the role of REST was first investigated by Trzaska et al. (2008) with the goal to obtain mature dopaminergic neurons. Indeed, the down-regulation of REST with lentivirus has been demonstrated to promote neuronal commitment [102].

Knock-down of gene expression can be obtained by different ways and in this study, we retained the siRNA epigenetic strategy to silence REST (siREST) in MSCs as it specifically targets the expression of the gene of interest as presented above (Introduction and aims). To introduce siREST, we chose two nanocarriers which present all the advantages that we discussed above

(Introduction and aims). We focused on two innovative systems: lipid nanocapsules and solid span nanoparticles.

Concerning LNCs, previous studies demonstrated the possibility to encapsulate plasmid DNA to develop a gene therapy strategy [116]. For this, the DNA is complexed with cationic lipids by electrostatic interactions leading to formation of complexes called lipoplexes, which are added to other components of the LNC. This strategy has demonstrated the capacity of LNCs to transfect *in vivo* models of glioblastoma [117–119]. Strong of these results, the encapsulation of siRNA in LNCs was successfully performed [153] and this system was able to transfect melanoma and glioma cells *in vitro* [154,155]. Nonetheless, the instability over-time of this system needed to be improved. In this way, the optimization of LNC-siRNA had to be performed with two goals: improving the stability over time of LNCs and protecting the siRNA from denaturation. In any case this system provides a positive surface, which can easily interact with negatively charged cell membranes. Although it is well known that nanoparticles with a positive surface charge transfect very well, the risk of toxicity is higher with those systems. However, recent evidence proves that anionic nanocarriers can also be effectively internalized [156], and that the transfection efficiency is not only dependent on the surface charge but also on the particle size and composition [157,158].

So, to determine the optimal nanocarrier to transfect MSCs, we chose a second system: Solid span nanoparticles (SP) to compare with LNCs. SP based on sorbitan monooleate (Span® 80) have been specifically adapted to provide effective siRNA association. Previous studies demonstrated the efficiency of SP to associate DNA by incorporating to their composition positively charged molecules like oleylamine (OA) [159]. This fatty amine is employed in the creation of emulsions and other delivery systems [160]. Poly-L-arginine (PA), a cationic moiety broadly used in gene therapy approaches [161] was also evaluated. OA and PA were used to provide a positive surface charge for the SP nanoparticles, thus enabling the association with the negatively charged, nucleic acids thus resulting in Span® 80-oleylamine nanosystems (SP-OA) or Span® 80-poly-L-arginine nanosystems (SP-PA), with a characteristic net negative surface charge [122]. Their capacity to provide higher *in vivo* transfection levels than

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adeno-associated viral vectors (AAV2) has been recently reported [123]. In this work, we conserved SP-PA to transfect MSCs and further modified the SP nanoparticles in order to coat them with cationized pullulan (AP), a natural polysaccharide obtaining SP-AP, as a strategy to easily associate siRNA by electrostatic interaction [162].

In this chapter, to improve human stem cell transfection and efficiency control of the stem cell fate, we have designed, optimized, characterized and evaluated two different siRNA nanocarriers transporting siRNA.

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# **PUBLICATION N°2**

# **CHARACTERIZATION AND COMPARISON OF TWO NOVEL NANOSYSTEMS ASSOCIATED WITH SIRNA FOR CELLULAR THERAPY**

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## ABSTRACT:

To direct stem cell fate, a delicate control of gene expression through small interference RNA (siRNA) is emerging as a new and safe promising strategy. In this way, the expression of proteins hindering neuronal commitment may be transiently inhibited thus driving differentiation. Mesenchymal stem cells (MSC), which secrete tissue repair factors, possess immunomodulatory properties and may differentiate towards the neuronal lineage, are a promising cell source for cell therapy studies in the central nervous system. To better drive their neuronal commitment the repressor element-1 silencing transcription (REST) factor, may be inhibited by siRNA technology. The design of novel nanoparticles (NP) capable of safely delivering nucleic acids is crucial in order to successfully develop this strategy. In this study we developed and characterized two different siRNA NP. On one hand, sorbitan monooleate (Span<sup>®</sup>80) based NP incorporating the cationic components poly-L-arginine or cationized pullulan, thus allowing the association of siRNA were designed. These NP presented a small size (205 nm) and a negative surface charge (-38 mV). On the other hand, lipid nanocapsules (LNC) associating polymers with lipids and allowing encapsulation of siRNA complexed with lipoplexes were also developed. Their size was of 82 nm with a positive surface charge of +7 mV. Both NP could be frozen with appropriate cryoprotectors. Cytotoxicity and transfection efficiency at different siRNA doses were monitored by evaluating REST expression. An inhibition of around 60% of REST expression was observed with both NP when associating 250 ng/mL of siRNA-REST, as recommended for commercial reagents. Span NP were less toxic for human MSCs than LNCs, but although both NP showed a similar inhibition of REST over time and the induction of neuronal commitment, LNC-siREST induced a higher expression of neuronal markers. Therefore, two different tailored siRNA NP offering great potential for human stem cell differentiation have been developed, encouraging the pursuit of further *in vitro* and *in vivo* studies.

## GRAPHICAL ABSTRACT:



## **KEYWORDS**

Nanocarriers, siRNA, mesenchymal stem cells, neuronal differentiation, REST.

## **ABBREVIATIONS**

MSC: mesenchymal stem cells; siRNA: small interfering RNA; SP: Span 80<sup>®</sup>; PA: poly-Arginine; AP: ammonium pullulan / cationized pullulan; NP: nanoparticles; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane; LNCs: lipid nanocapsules; REST/NRSF: (Repressor Element-1 silencing Transcription /Neuron-Restrictive Silencing Factor-1)

## **1. INTRODUCTION**

Mesenchymal stem cells (MSC) are multipotent stromal cells, capable of self-renewal and able to differentiate into a diverse set of cells within the mesoderm lineage [1]. They can also be an interesting source of cells for brain regenerative medicine because they secrete tissue repair factors, possess immunomodulatory properties and, in some conditions, may differentiate towards the neuronal lineage [2–4]. In order to safely implant them in the brain parenchyma they should be pre-committed to a neuronal phenotype. However, protocols only using external growth factors to drive MSCs into specific lineages seem to be insufficient to obtain high percentages of differentiated cells [5,6]. A lentiviral-mediated RNA interference vector that down-regulates the expression of REST/NRSF (Repressor Element-1 silencing Transcription /Neuron-Restrictive Silencing Factor-1) has been recently used to promote MSC differentiation into neuronal cells, which exhibited neuron-like morphology and function [7]. Indeed, REST/NRSF is a repressor transcription factor functioning as a master negative regulator of neurogenesis by binding to a specific DNA domain named RE1 motif [8,9]. In non-neural cells and in pluripotent stem cells, REST is abundant and represses neuronal genes [10] Consequently, the loss of REST in neural progenitors resulted in an acceleration of neuronal and oligodendrocyte differentiation [11].

Nowadays, a transitory inhibition of targeted gene expression in cells may be obtained by small interference RNAs (siRNA) without affecting the genome. These siRNA are able to produce a partial or total extinction of targeted messenger RNA (mRNA) and protein levels by degrading the mRNA. This epigenetic modification is quite simple, does not need genetic manipulation, is transitory and is now quite well understood. Although current applications in stem cells remain largely restricted to studies on molecular pathways and signaling, siRNA can be also used as a biomedical strategy to improve survival, direct lineage-specific differentiation of stem cells or help maintain a desired phenotype [12,13]. Within this line, the inhibition of Noggin with siRNA was capable to induce new bone formation *in vivo* [14]. But the mode of delivery of these novel therapeutic agents is of crucial importance for an efficient *ex vivo* or *in vivo* therapeutic strategy.

In the last decade, two different approaches for siRNA delivery have been developed: viral and non-viral vectors. Non-viral vectors (also named nanoparticles (NP) or nanocarriers) have been formulated to associate and to efficiently and safely deliver nucleic acids both *in vitro* and *in vivo*, opening many possible applications. In addition, they have many advantages and potentialities, including large-scale manufacture, low toxicity and low immunogenicity, and the possibility to customize them to target specific cell types [7,8]. Initially, NPs were designed to have a positive surface charge, in order to improve the interaction with the cell membrane that has a negative surface, and to avoid electrostatic repulsion [9]. However, recent evidence proves that anionic NPs can also be effectively internalized [15], and that the transfection efficiency is not only dependent on the surface charge but also on the particle size and composition [16,17]. In this frame, our research group has reported a novel NP based on sorbitan monooleate (Span<sup>®</sup> 80) (SP) that can be prepared using a simple, one-step and easily scalable procedure [18]. SP is a component widely used in the pharmaceutical industry (generally recognized as safe (GRAS)) due to its non-ionic surfactant properties at low concentrations [19]. These NPs are able to transfect *in vivo* the retinal pigment epithelial cells with a similar efficiency as adeno-associated viral vectors [20]

Transfection techniques currently use packaging particles such as liposomes to facilitate the cellular uptake of RNAi. They have shown high biocompatibility and are available in the market such as lipofectamine<sup>®</sup>, oligofectamine<sup>®</sup>, ready to be used. However, they exhibit some disadvantages such as high positive zeta potentials destabilising cell membrane, low transfection efficiencies in human stem cells, and instability immediately after complexation [21]. Moreover, transitional action reported with these liposomal carriers is not sufficient on their own to have an effect over-time. *In vitro* liposome transfection efficiency is also affected by the type of culture media and cell plating density, and does not provide an efficient protection against lysosomal nucleic acid degradation [22]. Lipid nanocapsules (LNCs) consisting of a lipid liquid core of triglycerides and a rigid shell of lecithin and polyethylene glycol hydroxystearate, are formulated by a simple and easily industrialized solvent-free process based on the phase inversion of an emulsion [23,24]. They allow entrapping the liposomes within their core and were thus recently modified to encapsulate the siRNA complexed with DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), protecting the siRNA from degradation [25]. DOTAP lipids presenting a cationic charge, allow the fixation of siRNA by electrostatic force and the interaction with the cell membrane improving the cellular uptake [26–28]. Moreover, DOTAP and DOPE are able to destabilize lysosome's membranes by a proton sponge effect [29]. However, the stability of these LNCs should be improved.

The transfection efficiency of the siRNA delivered by NP is often affected by the proliferative activity of human stem cells, and varies widely depending on the type of target cells [30]. To ameliorate the transfection of human stem cells and efficiently induce a neuronal-like differentiation, we have designed a novel SP-based NP conveying REST and further developed LNCs incorporating siREST with a good stability over time. [20]. First, we have modified SP NPs with cationized pullulan (AP) as a strategy to easily associate siRNA by electrostatic interaction thus obtaining a novel NP. Pullulan is a non-toxic natural polysaccharide presenting non-immunogenic properties [31]. This FDA

approved component possesses a wide range of industrial applications including health care and pharmacy [31]. Secondly, we further developed LNCs, and evaluated different formulations of LNCs incorporating siREST in order to obtain a better stability. The siREST-NPs were characterized by dynamic light scattering, UV spectrophotometry and electron microscopic methods in order to improve the understanding of their organization and structure. After evaluation of the long-term storage and stability of siRNA-NPs over time, we tested the cytotoxicity of both systems on human (h)MSCs and, finally, we evaluated their ability to transfect hMSCs and drive the commitment of hMSCs towards a neuronal phenotype determined by expression of neuronal genes.

## **2. MATERIALS AND METHODS**

### **2.1 FORMULATION**

#### **2.1.1 Synthesis of pullulan derivate :**

Pullulan was chemically modified in order to obtain a positively charged derivative (ammonium pullulan, AP) by alkylation of the original polymer. Synthesis was performed based on a previously described methodology [32]: an aqueous solution (5 mL) of KOH (0.504g, 9 eq), was prepared in a round bottom flask, under stirring, at 60 °C, to which pullulan (500 mg) and 3.62 mL (4.09 g, 27 eq) of GTMAC were added. After 5 h, an equal amount of GTMAC was added to the mixture, which was allowed to react until the completion of 24 h. It was then diluted with 10 mL of miliQ water, allowed to cool down to room temperature, and neutralized with HCl (2M). The resulting solution was dialyzed for 3 days, the water being replaced every 24 h, and the modified polymer recovered by freeze drying.

#### **2.1.2 SP-PA and SP-AP nanoparticles associated with siRNA**

To produce the SP nanoparticles, a solution of 6.6 mg/ml of sorbitan monooleate (Span<sup>®</sup> 80) (Sigma, Saint-Quentin Fallavier, France) in ethanol (organic phase) was prepared and it was subsequently added under magnetic stirring to an aqueous phase, leading to the spontaneous nanoparticle formation

[18]. To produce SP-PA and SP-AP nanoparticles, Poly-L-arginine (PA) or ammonium pullulan (AP) were dissolved in Mili-Q water at 0.16 mg/ml. Finally, ethanol was removed under reduced pressure on a rotary evaporator and the nanoparticles were concentrated to a final volume of 10 ml. Nanoparticles were isolated by filtration-centrifugation (Amicon® Ultra-0.5 centrifugal filter Devices, Merck Millipore, Ireland). The genetic materials (siRNA REST and Scramble) were associated to the nanoparticles' surface at different concentrations (from 0.1mg/ml to 0.5mg/ml) by incubation with nanoparticles at a 1:1 (v:v) ratio (100-100 µl) under magnetic stirring at room temperature during 2 hours in order to obtain siRNA-SP-AP and siRNA-SP-PA nanoparticles.

### 2.1.3 Liposomes and lipoplexes

For liposome preparation, a cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) (Avanti® Polar Lipids Inc., Alabaster, AL, USA), solubilized in chloroform, was weighted at a 1/1 molar ratio with the neutral lipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) (Avanti® Polar Lipids Inc., Alabaster, AL, USA) to obtain a final concentration of 30 mM of cationic lipid, (considering the number of charges per lipid molecule), i.e 1 for DOTAP. After chloroform evaporation under vacuum, deionized water was added to rehydrate the lipid film overnight at 4 °C which was sonicated the day after during 30 min.

Lipoplexes were formulated by simple equivolumar mix of siRNA and liposomes. This complex is characterized by the charge ratio [27] corresponding to the ratio between positive charge of lipids and negative charge of nucleic acids at 5.

### 2.1.4 siRNA-LNCs

LNCs were formulated, as described previously [23] by mixing 20 % w/w Labrafac® WL 1349 (caprylic-capric acid triglycerides, Gatefossé S.A. Saint-Priest, France), 1.5 % w/w Lipoid S75-3® (Lecithin, Ludwigshafen, Germany), 17 % w/w Kolliphor® HS 15 ( Polyethylene glycol-15-Hydroxystearate HSPEG BASF, Ludwigshafen, Germany), 1.8 % w/w NaCl (Prolabo, Fontenay-sous-

Bois, France) and 59.8 % w/w water (obtained from a Milli-Q system, Millipore, Paris, France) together under magnetic stirring. Briefly, three temperature cycles between 60 and 95 °C were performed to obtain phase inversions of the emulsion. A subsequent rapid cooling and dilution with ice cooled water (1:1.4) at the last phase inversion temperature (PIT) led to blank LNC formation.

To obtain siRNA LNCs, the water introduced at the last PIT was replaced by lipoplexes, i.e. REST siRNA: (sense sequence: 5'- CAG-AGU-UCA-CAG-UGC-UAA-GAA -3'; Eurogentec, Seraing, Belgium) and control (scrambled) siRNA (sense sequence: 5'- UCUACGAGGCACGAGACUU-3'; Eurogentec, Seraing, Belgium) complexed with cationic liposomes in a defined charge ratio as described above.

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To avoid the possible denaturation of siRNA by the high temperatures, the addition of lipoplexes at 75°C or 40°C was tested within the classical formulation. Two other formulations were performed, one with reduce PIT by increasing the NaCl concentration to 8% in the formulation and the last one without lipoid®.

## 2.2 CHARACTERIZATION OF BOTH NANOPARTICLES

### 2.2.1 Size, zeta potential, transmission electron microscopy and cryo-transmission electron microscopy

Size and zeta potential of LNCs and SP were measured by Dynamic Light Scattering (DLS) method using a Malvern Zetasizer® apparatus (Nano Series ZS, Malvern Instruments S.A., Worcestershire, UK) at 25 °C, in triplicate, after dilution in a ratio of 1:100 with deionized water. These parameters were followed daily during one month.

The morphological examination of siRNA-SP-AP was conducted by transmission electron microscopy (TEM) (CM 12 Philips, Eindhoven, The Netherlands). Samples were placed on copper grids with carbon films (400 mesh) (Ted Pella, USA) and stained with 2% (w/v) phosphotungstic acid solution for TEM observation.

The morphological examination of siRNA-LNC for cryo-TEM imaging were prepared using a cryo-plunge cryo-fixation device (Gatan, Pleasanton USA) in which a drop of the aqueous suspension was deposited on a carbon-coated grid (Ted Pella Inc., Redding, USA). The TEM grid was then prepared by blotting the drop containing the specimen so that a thin liquid layer remained across the holes of the carbon support film. The liquid film was vitrified by rapidly plunging the grid into liquid ethane cooled by liquid nitrogen. The vitrified specimens were mounted in a Gatan 910 specimen holder (Gatan, Pleasanton, USA), which was inserted into the microscope using a CT-3500-cryotransfer system (Gatan, Pleasanton, USA), and cooled with liquid nitrogen. TEM images were obtained from specimens preserved in vitreous ice and suspended across a hole in the supporting carbon substrate

### 2.2.2 [Encapsulation efficiency \(EE\)](#)

*Qualitative BET electrophoresis detection* - The EE and the integrity of siRNA molecules after the process of nanoparticle formulation were evaluated by agarose gel electrophoresis. A volume of nanoparticle suspension equivalent to 2.5  $\mu\text{g}$  of siRNA before and after treatment with Triton® X100 (Sigma, Saint-Quentin Fallavier, France) was mixed with gel-loading solution (Sigma, Saint-Quentin Fallavier, France) and disposed in each well of 1% agarose gel containing ethidium bromide (Sigma, Saint-Quentin Fallavier, France) [12]. Free siRNA in solution corresponding to 2.5  $\mu\text{g}$  constituted the controls. Samples were let to migrate 40 min at 50 V and 10 min at 90V in a Tris–EDTA buffer (Sigma, Saint-Quentin Fallavier, France).

*Quantitative UV detection* – A spectrophotometric method based on the study recently reported by David *et al.*, (2012), was used to evaluate the EE for LNC. Briefly, siRNA LNCs were mixed with chloroform and water to separate respectively hydrophilic and lipophilic components, sodium hydroxide was

added to destabilize lipoplexes and finally absolute ethanol to destroy LNCs [28]. After multiple centrifugations, four compartments were obtained: free siRNA, free lipoplexes (i.e. siRNA associated with liposomes), encapsulated siRNA and encapsulated lipoplexes within LNCs.

siRNA and SP-AP were separated from supernatant using Nanosep® Omega 30 kD microcentrifuge filters (Pall Corporation, Ann Arbor, USA) and siRNA was measured in the supernatant. To determine the concentration of siRNA, optical density of each sample was determined at 260 nm by UV spectrophotometer (UV-2600, Shimadzu, Noisiel, France) in triplicate conditions. The EE was determined by dividing the experimental drug loading by the theoretical drug loading. EE was followed every week during one month by these two methods.

### 2.2.3 [Encapsulated siRNA stability](#)

In order to determine the stability of the encapsulated siRNA during the transfection, a spectrophotometric method based on the study recently reported by David *et al.*, (2012), was used to evaluate the encapsulated si-RNA of LNCs. Briefly, siRNA LNCs were dissolved at a ratio of 1:10 in Opti-MEM® media (Life technologies, France) to mimic the transfection condition at two temperatures: 4°C (to mimic the storage condition) and 37°C (to mimic transfection condition in the incubator). Methods used to separate the different phases were described earlier. The encapsulated siRNA was determined by dividing the experimental drug loading by the theoretical drug loading and taking into account the dilution. It was followed at different times: 0, 4, 8, and 12 h after formulation. To confirm results, the integrity of siRNA was evaluated by agarose gel electrophoresis in the same conditions.

### 2.2.4 [Freezing](#)

The long-term storage assessment of siRNA-LNCs and siRNA-SP-AP was carried out by freezing at -20°C. The siRNA-LNCs and siRNA-SP-AP were frozen with phosphate buffered saline (PBS) (control solution) or with an aqueous solution of trehalose or of glucose as cryoprotectants in a 1:1 (v/v) ratio nanoparticles:cryoprotectants with a range from 1% to 15%. They were

then analyzed after 1-month storage at  $-20^{\circ}\text{C}$  by evaluating size and zeta potential measures by Dynamic Light Scattering (DLS) method using a Malvern Zetasizer® apparatus (Nano Series ZS, Malvern Instruments S.A., Worcestershire, UK) at  $25^{\circ}\text{C}$ , in triplicate, after dilution in a ratio of 1:100 with deionized water. The integrity of siRNA molecules after storage at  $-20^{\circ}\text{C}$  of nanoparticle formulations was evaluated by agarose gel electrophoresis.

### 2.3 MENSENCHYMAL STEM CELL (MSC) ISOLATION AND EXPANSION:

Whole bone marrow was obtained from the iliac crest of 15-yr-old (#34984) living male donor (Lonza, Walkersville, MD). Cells were seeded with  $\alpha$ -MEM : BE 12169F (LONZA) supplemented with 10% FBS, L-Glutamine 2mM (Sigma-aldrich, France, G7513) FGF 1 ng/ml, Heparin 5  $\mu\text{g/ml}$ , 1% penicillin-streptomycin (Sigma, Saint-Quentin Fallavier, France). The cells were maintained at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  humidified atmosphere. Passage numbers between 5-7 were used for the following experiments.

### 2.4 MSC TRANSFECTION

Experiments were performed in 1mL of MSC Opti-MEM® media (Life technologies, France). Oligofectamine® (Life technologies, France), which served as a positive control, was used with 100 ng or 250 ng of siRNA. SiRNA-LNCs were filtered with a 0.2  $\mu\text{m}$  filter (Acrodisc PALL GHP, VWR, Radnor, USA). siRNA-LNCs and siRNA-SP-AP were incubated with cells at  $37^{\circ}\text{C}$  in a humidified atmosphere with 5 %  $\text{CO}_2$  for 4h before serum addition, following Oligofectamine–siRNA protocol. Cells were harvested after appropriate time and assayed for mRNA expression levels by RT-qPCR and protein expression levels by western blot.

### 2.5 IN VITRO VIABILITY:

Cytotoxicity assays were performed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) (Promega, Madison, USA). and AlamarBlue<sup>R</sup> (resazurin) (Invitrogen). Twenty-five thousand cells, cells were transfected in a 24-well plate with the siRNA-REST and siRNA scramble with increasing concentrations of siRNA (0.05, 0.1, 0.25,

0.5 and 1 mg/mL). To determine the IC<sub>50</sub>, cells were subsequently cultured during two days and 20 µL of MTS/well was added and plates incubated 2.5 h at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>. The OD was evaluated by Mutliskan Ascent (Labsystems, Fisher Scientific, Wilmington, USA) at 492 nm. For the AlamarBlue assay, 10% AlamarBlue assay was added in RPMI media without serum and phenol red. After 4h of incubation, the fluorescence was read using a SpectraMax fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) at 530/590 excitation/emission wavelengths. Three replicates were used for each treatment.

Furthermore, cell numbers were also counted after each transfection performed. Forty-eight hours after transfection 200.000 cells were resuspended by trypsin (0.5 g porcine trypsin, 0.2 g EDTA; Sigma, Saint Louis, USA), washed twice with PBS and trypan blue staining was used to count the number of cells per well.

## 2.6 RNA EXTRACTION, RT-QPCR :

These experiments were performed following the guidelines of the PACEM core facility ("Plate-forme d'Analyse Cellulaire et Moléculaire", Angers, France). Sense and antisense desalted primer pairs (Eurofins MWG Operon, Ebersberg, Germany) were mixed in RNase free water at a final concentration of 5 µM (Table 1). Total RNA of cells were extracted and purified using RNeasyMicrokit (Qiagen, Courtaboeuf, France), and treated with DNase (10 U DNase I/µg total RNA). RNA concentrations were determined using a ND-2000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA) and used for normalization of the input RNA in the RT. First strand complementary DNA (cDNA) synthesis was performed with a SuperScript™ II Reverse Transcriptase (Invitrogen), in combination with random hexamers, according to the manufacturer's instructions. Following first-strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 40 µL RNase free water (Gibco). 2,5ng of cDNA was mixed with Maxima™ SYBR Green qPCR Master Mix (Fermentas) and primer mix (0.3 µM) in a final volume of 10µL. Amplification was carried out on a Chromo4 thermocycler (Biorad) or LightCycler 480 (Roche) with a first denaturation step at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 30 s. After

amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. Several housekeeping genes, Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Beta-2-microglobulin (B2M), Beta actin (Actb), and Heat shock 90 kDa protein 1 beta (Hspcb) were tested for normalization. The GeNorm™ freeware (<http://medgen.ugent.be/~jvdesomp/genorm/>) was used to determine that GAPDH and ACTB were the two most stable housekeeping genes. The relative transcript quantity (Q) was determined by the delta Cq method  $Q = E^{(Cq_{min} - Cq)}$  in all the samples tested - Cq of the sample), where E is related to the primer efficiency (E=2 if the primer efficiency=100%). Relative quantities (Q) were normalized using the multiple normalization method described in Vandesompele et al [33].  $Q_{normalized} = Q / (\text{geometric mean of the three most stable housekeeping genes } Q)$ . The  $2^{-\Delta\Delta Ct}$  method was retained, using the housekeeping genes and gene of interest tested on control sample and treated sample [34].

Gene	Full name	NM accession number	sequences
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase	<a href="#">NM_001289745.1</a>	Fwd: CAAAAGGGTCATCATCTCTGC Rev: AGTTGTCATGGATGACCTTGG
<b>ACTB</b>	Actin	<a href="#">NM_001101.3</a>	Fwd: CCAGATCATGTTTGAGACCT Rev: GGCATACCCCTCGTAGAT
<b>B3-TUB</b>	Tubulin beta 3 class III	<a href="#">NM_006086</a>	Fwd: CCAGTATGAGGGAGATCG Rev: CACGTACTTGTGAGAAGAGG
<b>REST</b>	RE1-silencing transcription factor	<a href="#">NM_001193508.1</a>	Fwd: ACTCATACAGGAGAACGCC Rev: GTGAACCTGTCTTGCATGG
<b>SCG10</b>	stathmin 2	<a href="#">NM_001199214.1</a>	Fwd: TGTCACTGATCTGCTCTTGC Rev: AGAAGCTAAAGTTCGTGGGG
<b>NFM</b>	Neurofilament, medium polypeptide (Variant 1)	<a href="#">NM_005382.2</a>	Fwd: GACCTCAGCAGCTACCAG Rev: TAGTCTCTTCACCCTCCAG

**Table 2. Sequence of primers validated in RT-qPCR**

## 2.7 WESTERN BLOT:

Total proteins were isolated by sonication of cells in lysis buffer composed of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8, 2.5 mM EGTA, pH 7.4, 0.1% Tween 20, 10% glycerol, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM  $\beta$ -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Roche). The quantification of protein used was performed according to Bradford method (26).

Twenty  $\mu$ g of protein extracts were separated by SDS-10% PAGE and transferred to PVDF membranes (GE Healthcare, VWR, Milan, Italy). The membranes were incubated overnight with rabbit anti-REST antibody (1/200) (Abcam, Cambridge, UK), washed with Tris-buffered Saline and incubated with anti-rabbit (1/5000) (32460, Thermo scientific) for 6 hours. Immunostaining was revealed by the Immuno-Star HRP substrate (BioRad, Segrate (MI), Italy)

according to manufacturer's instructions and quantified by Kodak Image Station 440CF. The image analysis was performed using the Kodak 1D 3.5 software (27). To confirm that equal amounts of protein were loaded membranes were incubated with anti- $\beta$  actin (Sigma-Aldrich, Italy) and revealed in the same way.

## 2.8 STATISTICAL ANALYSIS:

Three independent biological replicates were performed for all experiments described in this paper. Comparisons between all groups, supposed with normal distribution, were performed using a classical analysis of variance (one-way ANOVA) followed by a Tukey's *post-hoc* analysis. The encapsulation efficiencies for modified LNCs and non-modified LNC were compared using a t-test. Statistical significance was ascribed to a threshold p-value of 0.05 (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

## 3. RESULTS

### 3.1 SP NANOPARTICLES: SELECTION OF SURFACE COMPOSITION AND siRNA DOSE.

Using SP we can spontaneously form negatively charged nanoparticles with a size of about 140 nm and a zeta-potential of - 17 mV, being these nanoparticles stable during at least 1 month (**Figures 1A and 1B**). However, the negative surface charge of this nanocarrier renders a stable interaction with the negatively charged siRNA difficult to accomplish. In order to modify the surface charge of these SP nanoparticles and to easily associate the siRNA to the nanoparticle surface we evaluated the effect of incorporating on their composition two different cationic molecules, poly-L-arginine (PA) and ammonium pullulan (AP) to the SP formulation. The developed SP-PA and SP-AP blank nanoparticles (no siRNA associated) showed a nanoparticle size of 180 nm and 140 nm, respectively, which did not vary much from the SP nanoparticles. They also presented appropriate positive surface charges of

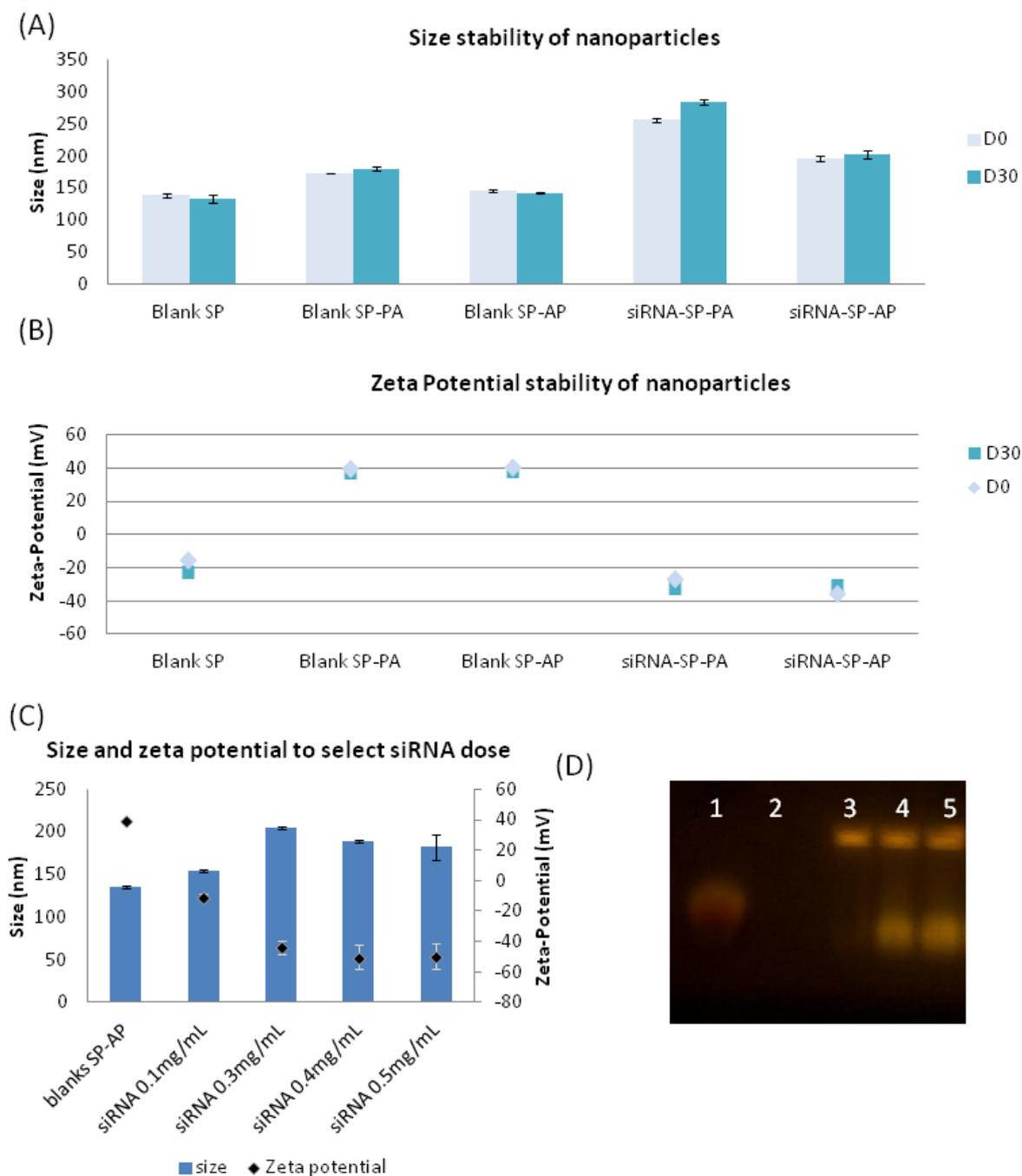
+40mV and +38mV, respectively (**Figure 1B**). No change in terms of size or zeta potential were observed when NPs were conserved for 1 month at 4°C (**Figures 1A and 1B**). Once developed, we complexed each system with the siRNA-scramble and the siRNA REST by simply mixing them together. Similar sizes and zeta-potentials were observed with both siRNAs. At day zero, the increase in size and the decrease on surface charge for siRNA-SP-PA NPs (280 nm and -37 mV) and siRNA-SP-AP NPs (205 nm and -38mV) (**Figures 1A and 1B**) indicated an effective electrostatic interaction between siRNA and the nanoparticles. On the contrary, when combining AP and siRNA to form AP-siRNA complexes as a control formulation a broad-range particle size distribution was observed, being characterized by the presence of aggregates rather than nanosystems. We also performed a stability study with siRNA-SP-PA and siRNA-SP-AP during 30 days. siRNA-SP-AP and siRNA-SP-PA were both perfectly stable in terms of size and in surface charge (**Figures 1A and 1B**). We selected SP-AP for the rest of experiments because pullulan is a non-toxic FDA approved natural polysaccharide presenting non-immunogenic properties with very interesting characteristics. Indeed, pullulan may be chemically modified in order to afford either hydrophobized or cationized derivatives, the former with the ability to carry hydrophobic molecules, and the latter showing high affinity towards DNA and RNA [35].

To determine the most favorable siRNA concentration capable of interacting with these NPs, we have tested different concentrations of siRNA ranging from 0.1 mg/ml to 0.5 mg/ml and checked the size and zeta-potential for each one. Similar particle sizes were obtained in this concentration range (around 190 nm). However, the surface charge decreased when the siRNA concentration increased, demonstrating the high capacity of AP to complex

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siRNA on the surface. In this case, we therefore speak of association efficiency. Above 0.3mg/ml of siRNA, we observed a stagnation of the surface charge (-45 mV) (**Figure 1C**) revealing a saturation of the system. This observation was confirmed by electrophoresis experiments performed to further assess the siRNA association ability of the developed SP-AP NPs. The absence of the typical bands of free siRNA in the formulations of NPs incubated with 0.1 and 0.3 mg/ml siRNA corroborated the effective association to the nanosystems in this concentration range (**Figure 1D**). At 0.3 mg/ml of siRNA the efficiency of association with the SP-AP was 33 % +/- 6 and we selected this dose for the subsequent studies.

Figure 1



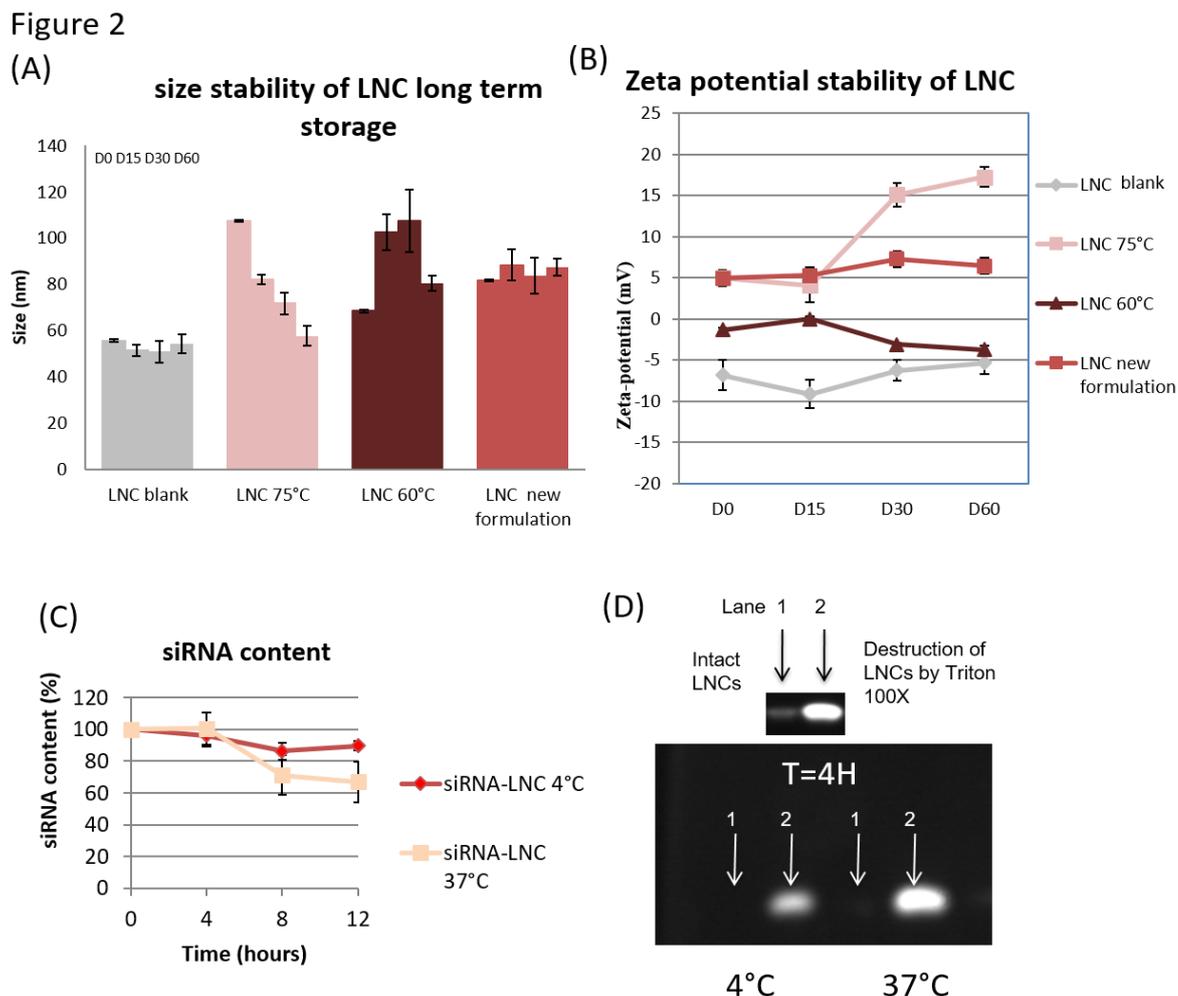
**Figure 1. Characterization of SP nanoparticles with different surface composition and siRNA dose:** (A) Size of blank nanoparticles based on SP, SP and poly-arginine (SP-PA) or SP and cationized pullulan (SP-AP), siRNA-SP-PA and siRNA-SP-AP at days 0 and 30 after storage (D0 and D30); (B) zeta-potentials at days 0 and 30 after storage; (C) Size and zeta-potentials of nanoparticles associating siRNA doses ranging from 0.1 to 0.5 mg/ml; (D) Electrophoresis experiments performed with free siRNA (Line 1) and siRNA-SP-AP associating different siRNA at different concentrations, such as (2): 0.1 mg/mL of siRNA; (3): 0.3 mg/mL; (4): 0.4 mg/mL and (5): 0.5 mg/mL. Free siRNA can be observed in the lines 4 and 5 illustrating the saturation on the association efficiency of the nanocarrier. Results are expressed as mean  $\pm$  standard deviation ( $n=3$ ). No difference was demonstrated on stability of SIRNA-SP-AP using ANOVA 1W, *post-hoc* Tukey.

### 3.2 OPTIMISATION OF siRNA-LNC

Initially, during siRNA-LNC formulation lipoplexes were added at the beginning of the last phase inversion temperature (PIT) (75°C), but an instability of the system has been observed [33]. To improve the stability and to avoid the possible denaturation of siRNA by the high temperatures this initial formulation was further modified. First, we increased the NaCl concentration to 8% thus obtaining a PIT at 60°C. In a second experiment, a novel formulation (modification of some components recently patented -ref: FR 4185991, 24 Sept 2014) was developed and the lipoplexes were also introduced at lower temperatures (40°C). Blank LNCs were used as control because their size and zeta potential were perfectly stable for 2 months (**Figures 2A and 2B**). We first noticed a difference in size (increased size) and zeta potential (increased surface charge) when comparing blank-LNC and the siRNA-LNCs formulations developed under the different experimental conditions (75°C, 60°C and 40°C), which suggest an effective encapsulation of lipoplexes in LNCs. The initial size (D0) of the siRNA-LNCs prepared at 75°C progressively decreased during a 2 months storage period from an initial value of 102 nm, whereas an increase of zeta-potential can be observed. These results suggest an instability and a rearrangement of the system (**Figures 2A and 2B**). The same behavior was observed with the formulation having a PIT at 60°C. There was an increase in size followed by a sharp decrease at D60, as well as a slight decrease in zeta potential. The last formulation at 40°C had a size of 85 nm with a positive surface charge of +7 mV which was perfectly stable over-time (**Figures 2A and 2B**). To evaluate the encapsulation efficiency of siRNA in LNCs electrophoresis experiments were performed. A low fluorescence band may be visible, indicating an incomplete encapsulation. The encapsulation yield determined by UV spectrophotometry was 43% +/- 7%, confirming that approximately half of the siRNA is stably incorporated within the LNCs. As in the case of SP-AP nanoparticles, generally no siRNA was visible probably due to free liposomes outside LNC able to complex and protect the siRNA.

The encapsulation efficiency was also evaluated after storage at 4°C under the conditions subsequently used in the transfection studies (37°C, 4h of incubation in Opti-MEM), in order to determine the stability of the encapsulated

siRNA. No differences were observed in both conditions at 0h and at 4h and no significant release was observed at 37°C in the 4h and 12h period.

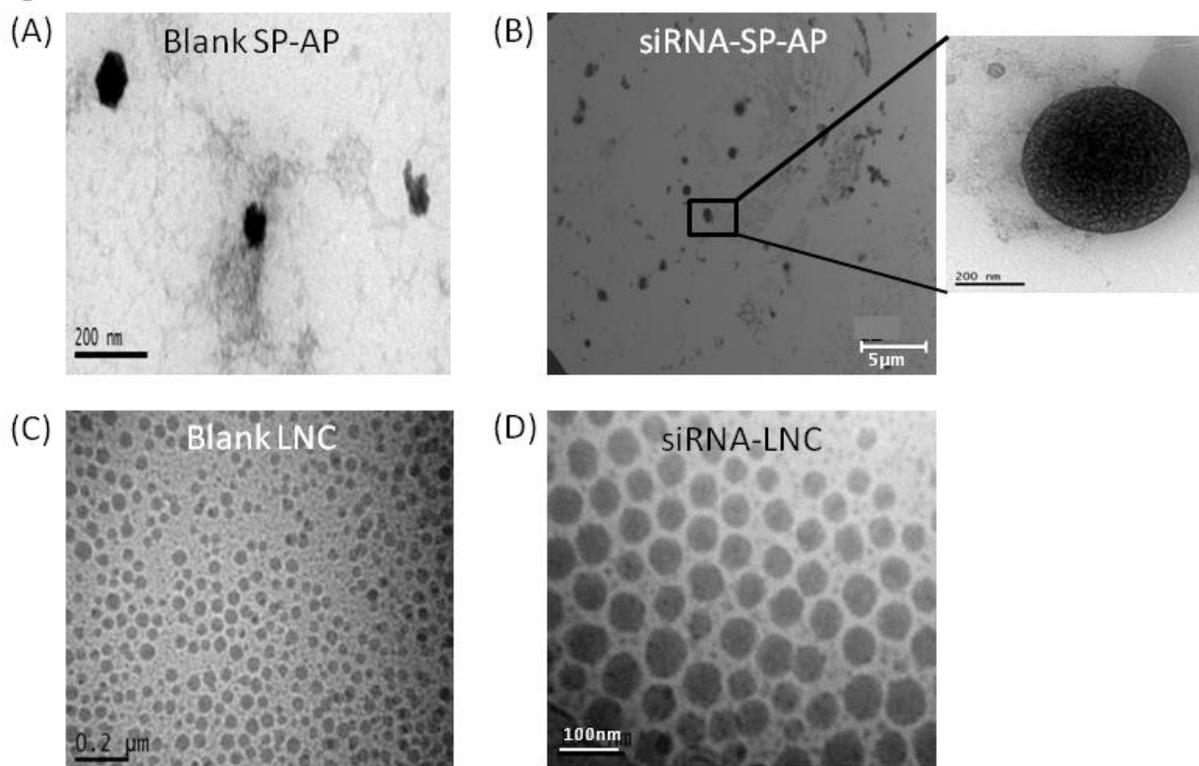


**Figure 2. Optimization of different siRNA-LNCs.** Size (A) and zeta potential (B) at different storage times (D0, D15, D30 and D60) showing a long-term stability for siRNA LNCs 40°C. EE evaluation by UV spectrophotometry of siRNA in LNCs in the Opti-MEM® at 4 and 37°C, showing no release during the first 4h (C), further confirmed in the electrophoresis experiments (D). Results (n=3) are expressed as mean measure  $\pm$  standard deviation and the encapsulation efficiencies were compared using a t-test.

### 3.3 MORPHOLOGICAL COMPARISON BETWEEN BOTH NANOCARRIERS

The morphology of SP-AP and LNCs was observed by transmission electronic microscopy (TEM) and cryo-TEM, respectively. The images illustrate the homogeneity of each nanocarrier population (**Figure 3**) and are in accordance with the sizes obtained by the light scattering technique for both siRNA-SP-AP (200 nm) and siRNA-LNC (80 nm) (**Figures 3B and 3D**).

Figure 3



**Figure 3.** Morphological comparison between SP-AP and LNC observed by transmission electronic microscopy (TEM) for Blank-SP-AP (A) and siRNA-SP-AP (B), and by cryo-transmission electronic microscopy (cryo-TEM) for Blank-LNC (C) and siRNA-LNC (D) showing homogeneous populations of spherical nanocarriers of around 200 nm (SP-AP based nanosystems) or 80 nm (LNC nanosystems).

### 3.4 siRNA-NANOPARTICLE FROZEN STORAGE.

**Table 2** summarizes the NPs size, zeta potential and polydispersity of the LNC and SP-AP formulations after 1 month storage at  $-20^{\circ}\text{C}$ . The frozen NPs can be easily resuspended by gentle shaking. However, in order to standardize the redispersion conditions, the particle size was determined after redispersing the frozen samples by vortex stirring. siRNA-LNCs were perfectly conserved with 1% of glucose without size or zeta-potential modification and si-RNA-AP could be frozen independently with 5% of glucose or trehalose, allowing integrity conservation of siRNA. The EE decreased of around 10% (from 43% to 33%) but the integrity of the siRNAs was still conserved for each nanocarrier as the siRNAs were still visible by electrophoresis after freezing and both NPs were able to transfect efficiently (data not show).

Simplex	Size (nm) before freezing	Zeta-Potential (mV) before freezing	Size (nm) after freezing	Zeta-Potential (mV) after freezing
siRNA-LNC PBS	81,7 +/- 2,45	4,929 +/- 0,82	6000	ND
siRNA-LNC 1% trehalose	83,9 +/- 3,15	5,35 +/- 1,56	95 +/- 4,72	17,2 +/- 6,18
siRNA-LNC 1% glucose	82,34 +/- 1,87	7,23 +/- 2,27	80,6 +/- 0,59	6 +/- 0,25
siRNA-SP-AP PBS	195,64 +/- 3,90	-36,3 +/- 1,22	275 +/- 7,40	-14,56 +/- 5,3
siRNA-SP-AP 5% trehalose	202,65 +/- 2,39	-41,2 +/- 0,74	199 +/- 1,98	-27,1 +/- 1,04
siRNA-SP-AP 5% glucose	202,12 +/- 2,83	-36,7 +/- 3,2	179 +/- 2,57	-27,4 +/- 0,88

**Table 3.** Physicochemical characterization of siRNA-SP-AP and siRNA-LNC after storage at -20°C. Formulations were stored at -20°C during 1 month with PBS (control), trehalose or glucose (cryoprotectors). Size and zeta-potential were determined using DLS (Zetasizer, Malvern) before and after storage. Most relevant results were presented here. Results (n=3) are expressed as mean measure  $\pm$  standard deviation.

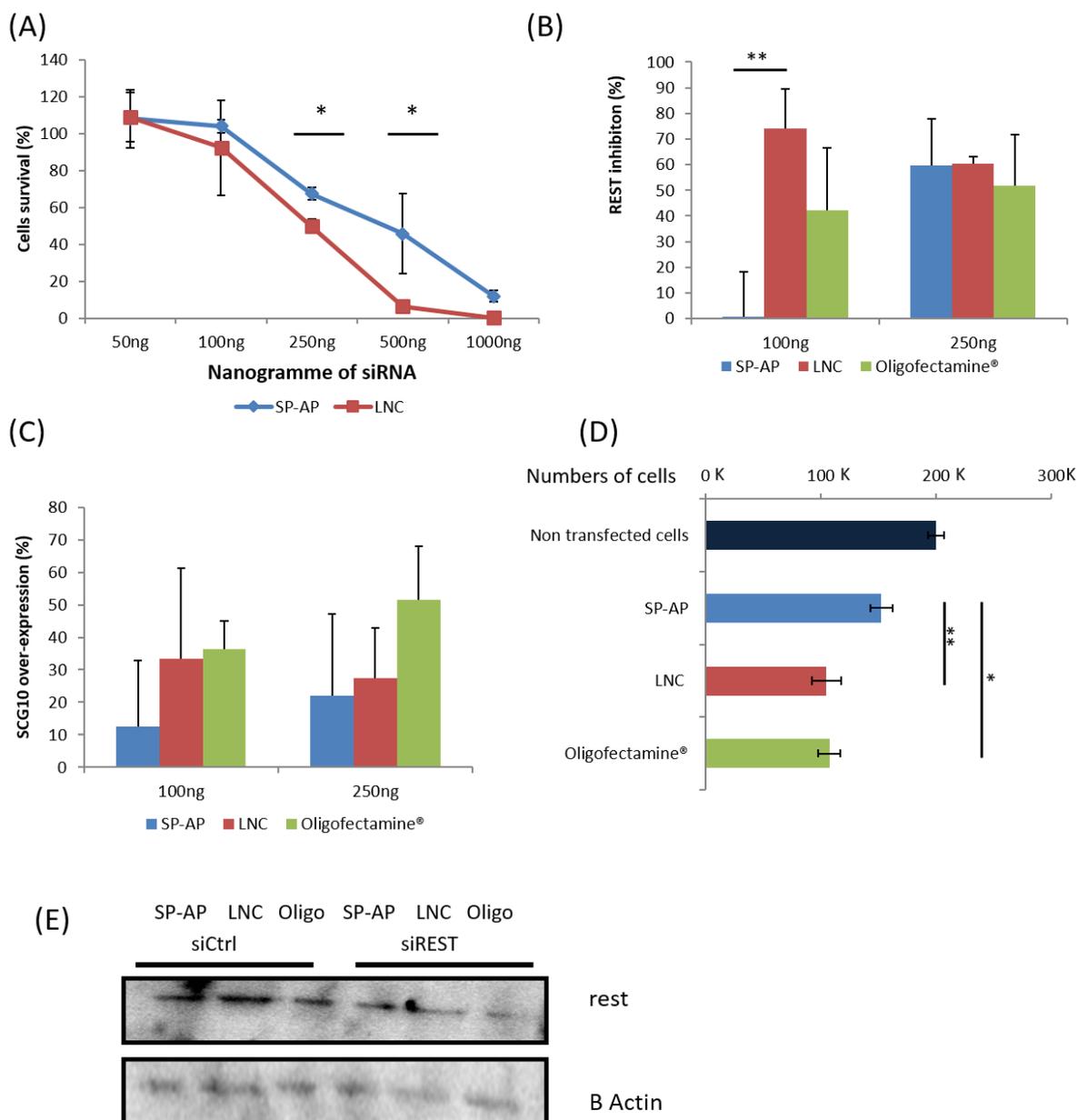
### 3.5 EVALUATION OF NANOCARRIERS TOXICITY AND SELECTION OF THE siRNA DOSE FOR MSC TRANSFECTION:

In order to study the toxicity of LNCs and SP-AP we evaluated the cell viability after 48 h exposure to different NPs concentrations expressed in terms of siRNA concentrations, ranging from 1  $\mu\text{g}/\text{mL}$  to 50  $\text{ng}/\text{mL}$ , that corresponds to a range of 60  $\mu\text{g}/\text{mL}$  to 1000  $\mu\text{g}/\text{mL}$  in the case of LNC nanosystems or 1  $\mu\text{g}/\text{mL}$  to 20  $\mu\text{g}/\text{mL}$  in the case of the SP-AP ones. A decrease in cell viability correlated to an increase in concentration of NPs was observed for both nanocarriers. No difference between both formulations was observed for 100  $\text{ng}/\text{mL}$  of siRNA. However, such differences can be appreciated at higher concentrations. Thus, in the case of LNCs, the most prominent decrease in cell viability was observed at 500  $\mu\text{g}/\text{mL}$  (corresponding to 500  $\text{ng}/\text{mL}$  si-RNA). At 250  $\text{ng}/\text{mL}$  and 500  $\text{ng}/\text{mL}$  of siRNA, SP-AP are significantly less toxic than LNCs (**Figure 4A**). On the basis of these results we selected siRNA doses of

100 and 250 ng for the initial transfection studies in human MSCs with siCtl and siREST (**Figure 4B**). The RT-qPCR analysis revealed that the down-regulation of REST seems to be dose-dependent for SP-AP in contrast to the LNCs, which showed an important effect at the lower concentrations assayed. Concretely, for LNC NPs a 77 % decrease of REST expression was observed using a siREST dose of only 100 ng, providing higher REST inhibition levels than the Oligofectamine<sup>®</sup> reagent that served as a positive control. A similar inhibition of REST expression of around 60% was observed with all the nanosystems when a concentration of 250 ng/mL of siRNA was evaluated. So, we selected this siRNA dose for further experiments. These experiments include the quantification of the SCG10 or, in other words, the expression of one direct target of REST. As we can appreciate in **Figure 4C**, a slight increase of SCG10 expression can be observed at 48 hrs after transfection when using both nanosystems at 250ng. The comparison of cell number 48 h after transfection with SP-AP, LNC and the Oligofectamine<sup>®</sup> reagent revealed the same profile with the LNCs and Oligofectamine (53 % of cell death) (**Figure 4 D**), while with SP-AP we observed only a 25 % of cell death. These results confirmed that SP-AP NPs are less toxic for hMSC.

To confirm the down regulation of gene expression provided by the different NPs we determined the protein knock-down by western blot analysis 36 h after transfection at a siRNA dose of 250 ng. In **Figure 4E** we can appreciate that REST protein was strongly inhibited with Oligofectamine<sup>®</sup> and LNC confirming the RT-qPCR data.

Figure 4



**Figure 4. Nanosystems cytotoxicity and siRNA dose selection for transfection studies:** cell survival for the SP-AP and LNC formulations assayed in human mesenchymal stem cells at increasing nanosystem concentrations (100% survival corresponded to 200 000 cells) (A), showing that SP-AP nanoparticles are the formulation with a lower toxicity at 250 ng/ml of siRNA. Quantification of REST inhibition by RT-qPCR 48 h after transfection with SP-AP and LNC, using the Oligofectamine reagent as positive control and si-Control (siCtrl) as negative control with each nanocarriers (REST expression was normalized to REST expression in MSC with siCtrl) (B), showing that REST was equally inhibited by both nanocarriers at a siREST dose of 250 ng/ml. Quantification of the overexpression of a direct target of REST (SCG10) (The SCG10 expression was normalized to SCG10 expression in MSC with siCtrl) (C). Cell counting 48h after transfection with the different formulations at a siRNA dose of 250ng/ml (D). Quantification of REST protein by western blot 56 h after transfection with the different formulations (250 ng/mL of siRNAs) to confirm the efficacy of REST knock-down (siCtrl was used as negative control and actin served as protein loading control) (mean REST expression  $\pm$  standard error of mean SEM (n=3)) (E). Statistical analysis performed with ANOVA 1W, *post-hoc* Tukey, \* p=0.05 \*\*p=0.005.

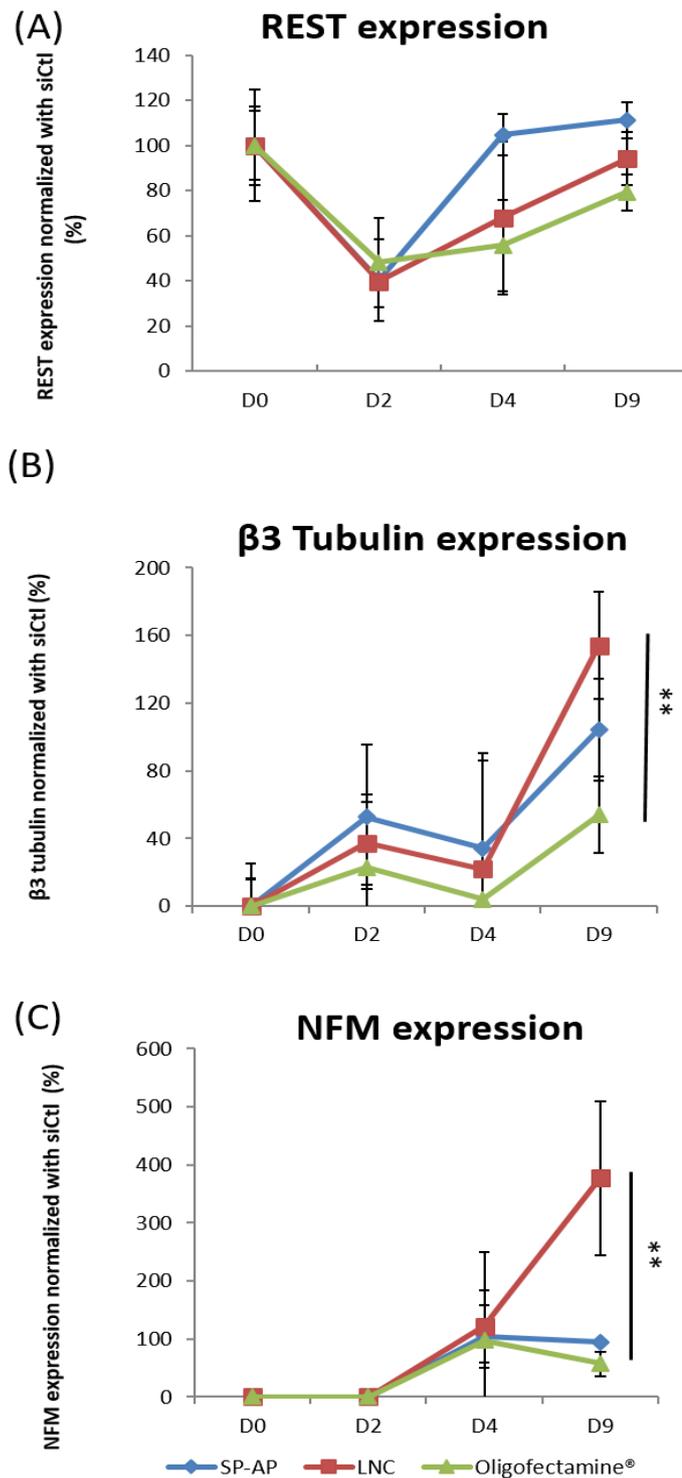
### 3.6 MESENCHYMAL STEM CELL DIFFERENTIATION

In order to determine the efficacy of siREST associated to the different formulations to drive neuronal differentiation over time, we quantified the mRNA expression for REST, his direct target (SCG10) and two neuronal markers:  $\beta$ 3-Tubulin ( $\beta$ 3-TUB) an early neuronal marker and a more mature neuronal marker, neurofilament medium polypeptide (NFM). MSCs were maintained in expansion media and not in differentiation media to exclusively evaluate the effect of REST inhibition.

The inhibition of REST was maximal at 48 h increasing progressively until day 9 for all nanocarriers (**Figure 5**). siREST-LNC was slightly more efficient compared to SP-AP over time and quite comparable to Oligofectamine® reagent used as positive control (**Figure 5A**), even when no statistically significant differences can be found.

On the other hand, although no real change was observed in SCG10 expression over-time (data not shown), the expression of  $\beta$ 3-TUB, which was already slightly expressed without treatment increased with both nanocarriers conveying siREST, particularly at D9 after transfection (until 160 % with siREST-LNC vs siCtrl-LNC), demonstrating the neuronal commitment induced by siREST. More interestingly, NFM expression appeared at day 4 and was four times more expressed with siREST-LNC than with siCtrl-LNC. Its expression was also higher in comparison with siREST SP-AP and oligofectamine at D9 (**Figure 5C**).

Figure 5



**Figure 5. Mesenchymal stem cells differentiation:** Transfection of hMSC was performed with 250 ng/ml of siControl and siREST. The expression of genes REST (A),  $\beta 3$  Tubulin, (B) NFM (C) was quantified at D0, D2, D4 and D9 after transfection with SP-AP, LNC and Oligofectamine®. Results were normalized with the expression of each gene with siControl. Results (n=3) are expressed as mean measure  $\pm$  standard deviation. Statistical analysis performed with ANOVA 1W, *post-hoc* Tukey, \* p=0.05 \*\*p=0.005.

## 4. DISCUSSION

For brain regenerative medicine, embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) have been proposed for transplantation therapy in human neuronal diseases. But the use of ESC or iPS present the risk of over-proliferation in the brain, in addition to the numerous ethical problems [34,35]. Research studies need to focus on resolving the choice of cell type. In order to preserve brain function in neuronal disorders, the graft of MSC is considered as an alternative therapeutic strategy. In this study we used MSCs as they are easily accessible, allow autologous grafting and present neuroprotective and tissue repair properties due to their paracrine activity [36]. Moreover, many clinical trials have been engaged worldwide in the last few years demonstrating their safety [37]. The versatile differentiation potentialities of MSCs have been demonstrated not only for various cells of mesodermal origin, but also for ectodermal origin such as neural/neuronal cells under defined culture conditions [36,3,38]. For brain protection and repair it is not necessary to obtain a mature neuronal phenotype establishing functional synapses with the surrounding cells, as the grafted cells will mainly function via a paracrine effect. However, they should present a neuronal-like phenotype to avoid any potential proliferation or non-desired mesodermal phenotype. In this study we demonstrated that a transitory inhibition of siREST, without altering their genome, is enough to induce neuronal commitment *in vitro* of MSCs. These pre-committed cells may thus be safely transplanted in the brain parenchyma and exert their tissue repair function [39].

Classically, growth factors and cytokines are added in the media to drive the differentiation of stem cells to neuronal-like progenitors [36,40]. The incorporation of a RNA interference approach to existing bio-chemical based differentiation protocols may provide an alternative synergistic approach to enhance the efficiency of directing stem cell fate. REST, a major negative regulator of several neuronal genes, thus repressing neuronal differentiation, is a target of choice for this approach [10]. Moreover, REST is differentially regulated throughout neuronal differentiation: highly expressed in embryonic stem cells, reduced in neural stem cells and neural progenitors [41] and largely

absent in mature neurons [8]. The inhibition of REST to improve neuronal differentiation has been previously evaluated by a permanent expression of siREST in mouse MSCs using a lentivirus carrying siREST [7], or by combining its inhibition with a cocktail of growth factors inducing neuronal differentiation in MSCs [42,43]. Our results are in agreement with these studies showing that the knock-down of REST induced a neuronal differentiation of MSCs [8]. However, in our hands, the sole inhibition of REST, without any other differentiation media, induced the neuronal commitment of hMSCs. Moreover, these NPs effectively delivered siREST to human stem cells which are not easily transfected and which generally show a high mortality rate [30,44]. The development of these biocompatible, large- scale manufactured NP (SP-AP and LNC) delivering siREST to hMSCs and inducing their neuronal commitment allow us to envisage this approach in human neuroregenerative medicine.

In this work, we have selected SP based nanocarriers on the basis of their demonstrated high *in vivo* transfection efficacy, being even higher than that provided by adeno associated viruses (AAV2) vectors [20]. For effective association between SP NP and siRNA we incorporated to these nanocarriers' two different cationic moieties: poly-l-arginine (PA) – a synthetic polyamino acid - and ammonium pullulan (AP) - a cationised polysaccharide-. Although PA was initially selected for its extensive use as a tool for gene delivery and due to its capacity for binding siRNA, we rejected PA based SP NP due to the resulting large particle size. We chose pullulan, due to its non-immunogenic, non-toxic, non-carcinogenic and non-mutagenic nature. Moreover, pullulan has been proposed for regenerative medicine and as a nanocarrier component, on the basis of its potential in the fields of immunization, gene delivery, and also in the design of imaging tools [45]. Although, pullulan is a promising polysaccharide for biomedical applications, the inherent neutral charge of this sugar does not allow its association with genetic materials [45]. To avoid this limitation we modified this polysaccharide and cationised pullulan (AP) was used as a component to be incorporated in SP nanocarriers [32] to efficiently transfect hMSC. Our results show that using AP we can develop homogeneous populations of SP-AP, which can be easily chemically modified in order to afford either hydrophobized or cationized derivatives, the former with the ability to carry hydrophobic molecules, and the latter showing high affinity

towards DNA and RNA. SP-AP NP effectively associated with the siRNA showing a spherical morphology that seemed more compact than that corresponding to LNCs. We have now managed to ameliorate the EE by optimizing the formulation of SP-AP thus obtaining 70% of siRNA complexation.

Concerning LNCs, these NPs were previously used for the encapsulation of lipophilic compounds, such as paclitaxel [46] and with MSCs as vehicles to deliver drugs into the brain [47]. LNCs were more recently adapted to the encapsulation of hydrophilic compounds (DNA, siRNA) [27,24,28]. However, stability over time for siRNA-LNC was not satisfying, especially in terms of size. In addition, due to their preparation requirements (phase inversion process at 75°C) the siRNA could be denatured during the association to these nanocarriers. However, recent experiments confirm the possibility to optimize these parameters. The optimization of the process (patent ref: FR 4185991, 24 sept 2014) and the diminution of the temperature down to 40°C led to LNCs with a long stable profile. Furthermore, the addition of siRNA after the phase inversion at 40°C avoids their denaturation, while preserving the characteristic positive charge on the surface of these nanocarriers. Recently, a new method of detection has been developed allowing the measure of the EE of siRNA-LNC which is now of 75%.

In this work we decided to evaluate the potential of transfection of the above described two types of nanosystems, which differ in the main properties affecting interaction with the biological media in general and with cells in particular (composition, particle size and surface charge). Surface charges play an important role in the internalisation within the cell [48]. Classically, NPs have been designed with a positive surface charge to interact favourably with the negatively charged phospholipid components of the cell membrane, but this interaction causes membrane cell damages and can be toxic [49]. This has been confirmed by this study, as the negatively charged span nanocarriers were only slightly toxic, whereas the higher toxicity of LNCs can be explained by a better cell interaction, internalisation and low cell density. Previous studies using LNCs and cancer cells did not show a high toxicity [28,33] because cell lines are more resistant than hMSCs and the density of cancer cells was ten times

more concentrated than in this study. Moreover, in a previous publication, we showed that MSCs can be internalized with higher doses of LNCs without any toxicity in 1h [50] and not in 4h as recommended in Oligofectamine's protocol. Indeed, in order to compare these NPs with a commercial reagent, we used the same protocol recommended in the Oligofectamine<sup>®</sup> guidelines. In addition, it is noticeable that the negatively charged SP-AP nanocarriers were able to transfect hMSCs with the same efficacy than the positively charged commercial reagent but avoiding cytotoxic events. Until recently, the internalisation of negatively charged NPs was controversial. Indeed, the internalisation of negatively charged NPs is believed to occur through nonspecific binding and clustering of the particles on cationic sites on the plasma membrane [51]. We here confirm the transfection ability of the negatively charged nanocarriers previously reported and discussed by our group [52]. Based on the literature we can suppose that SP-AP have the ability to undergo internalization via caveolae pathways, whereas cationic NPs such as LNC, commonly use the clathrin pathways [53–55].

The ability of a nanoparticulate system to stabilize various cargos from degradation and aggregation during storage represents a major advantage for its application. It is quite well known that aqueous suspension of NPs have a tendency to aggregate during long term storage [56]. In order to avoid this, we showed that the proposed NPs (LNC and SP-AP) could be frozen and stored while preserving the integrity of the associated siRNA. Nonetheless the NPs stability and genetic material association can be negatively influenced during freezing due to the ice crystals formed [57]. A suitable stabilizer, usually sugars (glucose, trehalose, mannitol...) can be used in the formulation protecting the physico-chemical properties of NPs and genetic materials [58]. On this respect, we initially thought that the pullulan-based nanocarrier might be frozen without cryoprotectant, due to its natural polysaccharidic nature, but we found that the cationization treatment seems to modify the cryoprotectant capacity of pullulan. In any case, the siRNA-SP-AP were easily frozen with different sugars (glucose and trehalose), conserving intact the associated siRNA. On the contrary, siRNA-LNCs conserved their size and zeta-potential only with glucose. Moreover, the loading efficiency after freezing and storage in glucose decreased less than 10% allowing using practically the same concentration of NPs. The observed increase

of the zeta-potential with trehalose was probably due to the loss of a fraction of the siRNA. Such features represent an advantage of LNC and SP-AP as compared to commercial transfection reagents. Indeed, several studies have demonstrated that lipoplexes (oligofectamine<sup>®</sup>) are not stable in liquid suspension for long-term storage [59,57], requiring their preparation immediately before use [60].

The two NPs effectively delivered siREST in a transient manner in hMSCs inducing a marked inhibition of REST resulting in a significant increase of neuronal markers (NFM and B3-TUB), which was maximal at day 9. This induced commitment of hMSCs to a neuronal phenotype in expansion media by an epigenetic approach using only synthetic Np is very encouraging. One other study reported the transfection of mouse neural progenitors with siREST and showed comparable levels of B3TUB expression at day 5 which was further increased at 2 weeks when a second transfection was performed to maintain REST inhibition [43]. However, in the cited study they added retinoic acid to better induce a neural differentiation. In our study we also observed with LNC-siREST a high increase of neurofilament levels, a mature neuronal marker, which was previously described for MSCs, but with a stable inhibition of REST using viral vectors [7]. The cell differentiation process occurs throughout time and requires a number of sequential events that lead from one cell differentiation state to another. Each new step is facilitated by the previous one, and our results show that only a transient inhibition of REST is necessary to engage the cells towards a neuronal-like phenotype. However, we didn't observe any significant change of SCG10 expression which is a direct target of REST and correlates with neurite out-growth [61]. Studies showing neurite out-growth and morphological changes use a stable inhibition of REST with lentivirus or shREST [7,42] which is not our case. The high level of NFM and B3-TUB observed in this work with LNCs, could be explained by a slow release of siRNA from these NPs. Indeed, LNCs remain at least 7 days in MSCs [50] compared to commercial reagents which are able to release siRNA during only 3-5 days [62]. A very fast siRNA delivery could saturate the endogenous microRNA (miRs) processing mechanisms or enter in competition with them. For example for the incorporation and retention in RNA-Induced Silencing Complex (RISC), which is essential for the silencing mechanism of both

interfering RNAs [63]. An over-expression of miR-124 increases neuronal (early and mature) markers in MSCs [64]. Furthermore, several miRs, such as miR-124, miR-132, miR-9, are essential for the neuronal differentiation and are direct targets of REST [65]. In our study, we can suppose that the slow release of siREST allows the cooperation with miRs to enhance the neuronal commitment. However, more experiments are necessarily to confirm this hypothesis.

For regenerative medicine studies, we can envisage the possibility to elicit a synergistic effect on neuronal commitment of stem cells by the combined delivery of a morphogen with a REST knock-down. This approach may be applied to SP-AP where siRNA is in the surface letting the core free. It could also be envisaged with LNCs, which have the capacity to associate different morphogens, such as retinoic acid [66]. It could be a useful strategy for generating functional neurons for therapeutic purposes and drive differentiation directly into the brain *in vivo*.

## **CONCLUSION**

In this study we designed and optimized two novel nanocarriers capable of safely associating and delivering nucleic acids: LNC and SP-AP. These systems were perfectly reproducible and could stand long time storage. We have demonstrated the capacity of both systems to knock-down REST and differentiate human stem cells towards a neuronal phenotype at least with the same efficacy of a commercial reagent. Therefore, these nanocarriers can be considered as promising platforms for the development of effective and safe gene based regenerative approaches.

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## DISCUSSION

The use of non-viral siRNA delivery seems to be a promising strategy to induce a neuronal MSC differentiation prior to cell transplantation for a regenerative and safe therapeutic strategy in HD. In this study we showed that MSCs were only induced to neuronal progenitors with the inhibition of REST which have already described in the literature to play a role in the neurogenesis [163]. Indeed, REST is highly expressed in ES cells but it is down-regulated to a minimal level in neural stem cells and neural progenitors [99,152]. After the differentiation of these progenitors, REST remains present in oligodendrocytes [164] and largely absent in neurons [152]. REST is crucial for maintaining the self-renewal of neural progenitors and the ratio of neurons. Indeed the loss of REST in neural progenitors resulted in an acceleration of neuronal and oligodendrocyte differentiation [165]. We demonstrated the neuronal commitment with the significant apparition of  $\beta$ 3-tubulin and NFM two days after transfection with siREST. Nonetheless, siRNA cannot cross cell membranes without modifications or carriers. So we have designed two nanocarriers LNC and SP-AP. In the design of such nanocarriers significant factors have been taking into account to overcome limitations associated with insufficient siRNA delivery, such as nanocarrier size, surface charge, shape, chemical composition, and stability of f nanocarriers [166].

In order to compare these nanosystems, we chose to transfect the same quantity of siRNA, which corresponded to different quantities of nanoparticles. As siRNA-SP-AP were non toxic we can easily imagine to transfect MSCs for a longer time, repeat the transfection or increase the dose to further induce the neuronal differentiation of these cells. These data are interesting because the major problem nowadays in transfection is the toxicity [167]. Indeed, MSCs are difficult to transfect without affecting their viability, resulting in very low efficiencies of transfection with range from 2 to 35% [168].

While siRNA-LNC had a better capacity to transfect MSC, siRNA-SP-AP had the same efficacy as a commercial reagent without any toxicity. The difference in terms of size (less than 100 nm for LNC, more than 100 nm for SP-AP), the composition (both are lipid based nanocarriers but their components

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are different), the surface charge (positive for siRNA-LNC, and negative for siRNA-SP-AP), as well as the localization of siRNA (entrapped in liposomes for siRNA-LNC and on the surface for siRNA-SP-AP) could explain the results observed.

Indeed, we can imagine that the positively charged siRNA-LNCs favorably interacted with the negatively charged phospholipid components of the cell membrane, and that this interaction caused membrane cell damages probably explaining their toxicity [169]. On the other hand, the negatively charged span nanocarriers were only slightly toxic. However, the better neuronal commitment with siREST-LNC can be explained by a better cell interaction and internalization demonstrated. Long-term conservation of those nanocarriers represents another advantage of LNC and SP-AP as compared to commercial transfection reagents or viral vectors [170,171]. Therefore, these nanocarriers can be considered as promising platforms for the development of effective and safe gene based regenerative approaches.

# CHAPTER III

## INTRODUCTION

Cell therapy strategies using mesenchymal stem cells in HD have been investigated for the last 20 years because MSCs secrete many tissue repair factors favorable for neuroprotection. The paracrine effect of MSCs on HD was investigated by Lee et al. (2009) in cell culture experiments and *in vivo* in two different rodent HD models [145]. Transplantation of MSCs into QA model of HD led to improvement in behavior and reduced the lesion volume. These beneficial effect could be explained by the secretion of neurotrophic factors including BDNF, CNTF, nerve growth factor, insulin-like growth factor 1 and epidermal growth factor [147]. Then similar results were obtained in genetic models of HD, YAC128, N171-Q82Q and BACHD, demonstrating the interest of the use of mesenchymal stem cells in this disease [145,146,172]. More importantly, these studies reveal the stimulation of neuronal differentiation of endogenous neural stem cells by MSCs due to the paracrine effect [146]. However, the lacks of consistent neuronal differentiation of transplanted MSCs have limited their therapeutic efficacy in slowing the progression of HD-like symptoms in animal models of HD.

MIAMI cells, which are a primitive and unique subpopulation of MSCs secreting more tissue repair factors than MSCs [94] seem to be a good alternative. MIAMI cells, have also been shown to have a neuroprotective effect after implantation in Parkinson's disease and we have already demonstrated their neuronal commitment using a pre-treatment with EGF (epithelial growth factor) and bFGF (Basic fibroblast growth factor) which induced a decrease in the expression of stem cells markers such as Oct4A, Notch1 and Hes5 and increased markers of neural precursors such as Nestin and  $\beta$ 3-tubuline. Recently, we showed in a rat model of Parkinson's disease (PD), that striatal implantation of EGF/bFGF pre-treated MIAMI cells had a neuroprotective effect leading to functional recovery. This effect was probably due to their secretion of GDNF, but also of VEGF as observed in an *ex vivo* model of PD [173]. A similar neuroprotective effect was also observed for MIAMI cells and EGF/bFGF pre-treated MIAMI cells in an *ex vivo* and in an *in vivo* model of cerebral ischemia [174]. PAMs, which can stimulate the survival and

differentiation of cells, were combined to MIAMI cells to evaluate their effect in these models. In both of the cerebral ischemia models as well as in the *ex vivo* model of PD, if the cells were adhered onto PAMs, an increased survival of the transported cells was observed after transplantation leading to an enhancement of their neuroprotective effects [84,174].

In addition, MIAMI cells can be induced towards a neuronal phenotype in a NT-3 dependant manner and into a dopaminergic phenotype using specific inductors (SHH and FGF8) *in vitro*. Moreover, a laminin substrate favours the neuronal differentiation of EGF/bFGF pre-treated MIAMI cells. The implantation of the E/F MIAMI pre-committed towards the dopaminergic phenotype and adhered onto laminin-coated PAMs (LM-PAMs) releasing NT-3 (LM-PAMs-NT-3) led to the protection/repair of the nigro-striatal pathway and to functional recovery of the PD rats. Moreover, the evaluation of these cells in the Parkinson organotypic slices, demonstrated the partial capacity of the E/F MIAMI cells adhered onto LM-PAM-NT-3 to differentiate into dopaminergic neurons [84]. Based on these promising results, we wanted to investigate the effect of PAMs delivering BDNF as neuroprotective strategy in HD. Indeed, BDNF has neuroprotective properties and sustain the physiological processes of normal intact adult brain [68] and more particularly for GABAergic striatal neurons.

In this Chapter, we described a new strategy to induce neuronal commitment using siREST, media, growth factors to improve the differentiation. Inspired from the literature, we tested different protocols to obtain GABAergic neurons. The implantation of cells adhered to PAMs releasing BDNF then has been realized into organotypic brain slices modeling HD.

*Publication in process*

## **PUBLICATION N°3**

**MIAMI NEURONAL COMMITTED CELLS**  
**COMBINED WITH PHARMACOLOGICALLY ACTIVE**  
**MICROCARRIERS: AN INNOVATIVE THERAPEUTIC**  
**STRATEGY IN HUNTINGTON'S DISEASE**

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## **ABSTRACT:**

Clinical trials using mesenchymal stem cells in the central nervous system are now underway, and are focused on the safety of these cells. Marrow-isolated adult multilineage inducible (MIAMI cells), a subpopulation of mesenchymal stem cells raises great interest for cell therapy in Huntington's disease (HD) because they possess immunomodulatory properties and tissue repair capacities. Moreover, they can be differentiated into neurons-like cells under specific conditions. In this study, we investigated the role of REST inhibition in the GABAergic differentiation of MIAMI cells. We designed an *in vitro* protocol combining siRNA-nanocapsules, substrates, media, and cytokines to push MIAMI to a GABAergic neuronal lineage. We further combined these cells to pharmacologically active microcarriers with a biomimetic coating of laminine and releasing brain derived neurotrophic factor (BDNF) to improve their survival and differentiation. These results show that siREST combined with media containing growth factors allowed differentiating MIAMI cells toward the neural lineage *in vitro*. These data are supported by the slow delivery during 6 days of siRNA on MIAMI cells due to the nanocapsules. The monitoring of gene expression of neuronal and GABAergic markers during the differentiation period suggested that GABAergic precursors and finally GABAergic neuronal-like cells were obtained. Importantly, those cells did not lose their paracrine effect. Indeed, the secretion of VEGFa and BDNF are interesting for the neuroprotection in HD. Finally, preliminary data shows that the combination of PAMs releasing BDNF improved the survival of cells and drives their differentiation. Ours results open the possibility toward cell based therapy for HD.

## **Keywords:**

Tissue engineering, Huntington's disease, siRNA, lipid nanocapsules, microcarriers.

## **Abbreviations:**

MSCs: Mesenchymal stem cells, siRNA: Small interfering RNA; NPs: Nanoparticles, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane; LNCs: Lipid nanocapsules; REST/NRSF: (Repressor Element-1 silencing Transcription /Neuron-Restrictive Silencing Factor-1); DARPP32: Dopamine- and cAMP-Regulated neuronal PhosphoProtein of 32kDa; GAD67: Glutamate decarboxylase 67; GSBS: Grey's Salt Balanced Solution; HD: Huntington's disease; HTT: Huntingtin gene; htt: protein huntingtin; MEM: minimum essential medium eagle, MSN: medium

spiny neurons; NeuN: neuronal nuclei; PBS: phosphate buffered saline; PFA: paraformaldehyde; PAMs: pharmacologically active microcarriers.

## 1. INTRODUCTION

Huntington's disease (HD) is a genetic disorder caused by the abnormal repetition of CAG in the Huntingtin gene which results in a pathological expansion of a polyglutamine (polyQ) tract in the huntingtin (htt) protein [1,2]. This neurodegenerative disorder is characterized by aggregation of htt in the brain, and more specifically in the striatum [3]. The neuropathological changes in HD are selective, and progressive degeneration of striatal GABAergic medium spiny projection neurons is observed [4]. Clinically, this results in involuntary movements, cognitive impairment and psychiatric manifestations [5]. Typically, HD eventually culminates in death around 15–20 years after the onset of motor symptoms [6]. Currently, there is no proven medical therapy to alleviate the onset or progression of Huntington's disease [7].

Cell replacement therapies in neurodegenerative diseases have been investigated for the last 30 years. Although the procedures are theoretically feasible, some limitations of the therapy still give cause for concern. At the beginning, fetal striatal tissues were transplanted to modify HD progression in humans [8,9]. Nonetheless, some favorable effects have been demonstrated but the difficulties in tissue availability and viability remain the major concern. Moreover, fetal tissue grafts do not alter the toxic effects of mutant huntingtin and has a high risk of rejection. There are also ethical arguments and concerns about contamination and heterogeneity of the tissues [10]. Recently, embryonic and induced pluripotent stem cells (ESC and iPS) were shown to successfully differentiate into medium spiny neurons [11–13]. But after human ESC transplantation into rat brains, tumor formation was reported. Mesenchymal stem cells (MSCs), have emerged for clinical transplantation studies due to their easy availability and their capacity to release neurotrophic factors and create a neuroprotective microenvironment [14]. Clinical trials using MSCs in the central nervous system (CNS) are now also underway for many neurological disorders and have shown the feasibility of this approach [8,10]. Pre-clinical studies with HD models have shown improvement in behaviour and reduced the lesion

volume. These beneficial effects could be explained by the secretion of neurotrophic factors including BDNF, CNTF, nerve growth factor, insulin-like growth factor 1 and epidermal growth factor [15,16].

The trans-differentiation of MSCs into a neural/neuronal lineage is possible but their functional maturity is still insufficient. The MSC must commit to a neuronal cell and maintain this phenotype and if possible try to replace the damaged neurons. In order to induce a neural/neuronal differentiation of MSCs the silencing of a master gene involved in neural specification named repressor element 1 (RE-1) silencing transcription factor (REST) [17,18] was obtained by a recombinant lentivirus. Nanoparticles have been formulated to associate and to efficiently and safely deliver siRNA both *in vitro* and *in vivo*. In particular, lipid nanocapsules (LNC) were recently modified to encapsulate the siRNA complexed to lipids into their core, thus protecting the siRNA from degradation. LNCs consisting of a lipid liquid core of triglycerides and a rigid shell of lecithin and PEG are formulated by a simple and easily industrialized solvent-free process based on the phase inversion of an emulsion [19,20]. They have a high stability and are able to destabilize lysosome's membranes by a proton sponge effect [21]. Recently, we demonstrated that LNCs associated with siREST in MSCs were able to induce their neuronal commitment with a better efficiency than a commercial reagent (Publication submitted). MIAMI cells, which are a primitive and unique subpopulation of MSCs secreting more tissue repair factors than MSCs [22] seem to be a good alternative because they can be induced differentiated in all three germ layers [23]. The demonstration of the capacity to MIAMI cells to differentiate into dopaminergic neurons presenting appropriate electrophysiological properties after commitment with EGF (epithelial growth factor) and bFGF (Basic fibroblast growth factor) have already done [24].

The major problem concerning cell therapy studies is the survival and engraftment of transplanted cells. Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor that is widely used in brain regeneration applications due to its ability to support the survival of neurons [25,26] and promote neuronal differentiation [27]. Moreover, in the case of HD, several studies demonstrated that the expression of BDNF is reduced in the patient's brains. Promising results show that the BDNF supplementation increases the survival of enkephalin-

immunoreactive striatal neurons, reduces striatal interneuronal loss and improves motor function in HD animal models [28–30]. Despite these promising results, the therapeutic delivery of human BDNF has raised a number of problems related to its, short *in vivo* half-life, poor availability resulting from its degradation after injection, and an uncertain passage through the blood-brain barrier (BBB) when administered in the periphery [31]. The delivery of therapeutic proteins requires microcarriers able to protect them and control their release over-time. In this way, pharmacologically active microcarriers (PAMs) developed by our laboratory are carriers constituted of PLGA a “Food and Drug Administration” approved polymer [32]. PAMs are biodegradable and biocompatible PLGA microspheres covered with ECM molecules, thus conveying cells on their biomimetic surface providing an adequate 3D microenvironment for the transplanted cells *in vitro* and *in vivo*. Recently, laminine substrate favours the neuronal differentiation of EGF/bFGF pre-treated MIAMI cells, and can integrate to the PAMs surface. Moreover, the encapsulation of protein during the formulation allows a controlled release of bioactive molecules. In this way, BDNF which is also involved in neuronal GABAergic differentiation and may maintain the differentiated phenotype of the transported cells could be encapsulated in PAMs. So PAMs represented an ideal microcarrier able to release active proteins [33–35] and providing a microcarrier for MIAMI cells due to their biomimetic surface [36].

In this study, we propose an innovative strategy for cell replacement in HD. Inspired by recent studies; a simple protocol will be tested to differentiate MIAMI cells into GABAergic neurons-like. In order, to improve the commitment, the transfection of siREST will be performed with LNC. Finally, we plan to combine PAMs releasing BDNF with pre-committed MIAMI cells and graft these complexes in an *ex vivo* model of HD to evaluate their impact on the regeneration of the lesioned striatal GABAergic cells.

## 2. MATERIALS AND METHODS

### 2.1 LNC FORMULATION

#### 2.1.1 Liposomes and lipoplexes

For liposome preparation, a cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) (Avanti® Polar Lipids Inc., Alabaster, AL, USA), solubilized in chloroform, was weighted at a 1/1 molar ratio with the neutral lipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) (Avanti® Polar Lipids Inc., Alabaster, AL, USA) to obtain a final concentration of 30 mM of cationic lipid. After chloroform evaporation under vacuum, deionized water was added to rehydrate the lipid film overnight at 4 °C. It was then was sonicated the day after during 30 min.

Lipoplexes were formulated by simple equivolumar mix of siRNA and liposomes. This complex is characterized by the charge ratio [37], corresponding to a ratio of 5 between positive charge of lipids and negative charge of nucleic acids..

#### 2.1.2 siRNA-LNC

LNCs were formulated, as described previously [19] by mixing 20 % w/w Labrafac® WL 1349 (caprylic-capric acid triglycerides, Gatefossé S.A. Saint-Priest, France), 1.5 % w/w Lipoid S75-3® (Lecithin, Ludwigshafen, Germany), 17 % w/w Kolliphor® HS 15 ( Polyethylene glycol-15-Hydroxystearate HSPEG BASF, Ludwigshafen, Germany), 1.8 % w/w NaCl (Prolabo, Fontenay-sous-Bois, France) and 59.8 % w/w water (obtained from a Milli-Q system, Millipore, Paris, France) together under magnetic stirring. Briefly, three temperature cycles between 60 and 95°C were performed to obtain phase inversions (PI) of the emulsion. A subsequent rapid cooling and dilution with ice cooled water (1:1.4) at the last phase inversion temperature (PIT) led to blank LNC formation.

To obtain siRNA LNCs, the water introduced at the last PIT was replaced by lipoplexes, i.e. REST siRNA: (sense sequence: 5'- CAG-AGU-UCA-CAG-UGC-UAA-GAA -3'; Eurogentec, Seraing, Belgium) and control (scrambled)

siRNA (sense sequence: 5'- UCUACGAGGCACGAGACUU-3'; Eurogentec, Seraing, Belgium) complexed with cationic liposomes in a defined charge ratio as described above.

### 2.1.3 Fluorescent siRNA-LNC-DiD

To formulate fluorescent siRNA LNCs, a solution of DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate; em. = 644 nm; exc. = 665 nm) (Invitrogen, Cergy-Pontoise, France) solubilized in acetone at 25 mg/mL was prepared.

DiD concentration was fixed at 200 µg/mL of LNC suspension or corresponding to 1,36 mg of DiD per grams of Labrafac<sup>®</sup> [38]. The adequate volume of DiD I solubilized in acetone was incorporated in Labrafac<sup>®</sup> and acetone was evaporated at room temperature. The formulation process was unchanged and formulation was stored at 4°C, protected from light. For siRNA fluorescent LNCs, a fluorescent Alexa488 siRNA (em. = 488 nm; exc. = 524 nm, Eurogentec) was used.

## 2.2 CHARACTERIZATION OF siRNA LNCs

### 2.2.1 Size and Zeta potential

The size and zeta potential of LNCs were measured by using the Dynamic Light Scattering (DLS) method using a Malvern Zetasizer<sup>®</sup> apparatus (Nano Series ZS, Malvern Instruments S.A., Worcestershire, UK) at 25 °C, in triplicate, after dilution at a ratio of 1:200 with deionized water.

### 2.2.2 Encapsulation efficiency

A spectrophotometric method was used to evaluate the encapsulation efficiency (EE %) as recently described [9]. Briefly, siRNA LNCs were mixed with chloroform and water to separate hydrophilic and lipophilic components, respectively. Sodium hydroxide was added to destabilize lipoplexes, and finally absolute ethanol was added to destroy the LNCs. After two centrifugations, four fractions were obtained: free siRNA, free lipoplexes, encapsulated siRNA and encapsulated lipoplexes into LNCs. To determine the concentration of siRNA, the optical density of each sample was read at 260 nm (UV-2600, Shimadzu,

Noisiel, France) in triplicate conditions and compared to a range curve to determine the ratio of the encapsulated siRNA per total siRNA detected in the formulation (EE%).

## 2.3 CELL CULTURE

### 2.3.1 MIAMI E/F

MIAMI cells were expanded in vitro from passage 4-5 on fibronectin (Sigma Aldrich, St Louis, USA) coated flasks at 125 cells/cm<sup>2</sup> in low oxygen tension in Dulbecco's Modified Eagle Medium-low glucose (DMEM, Gibco, Life Technologies, Paisley, UK), supplemented with 3% of serum, 30µg/ml ascorbic acid and a mixture of lipids (working concentration of 510nM lipoic, 70nM linolenic and 150nM linoleic acid, all from Sigma). Then a 10 day treatment with an addition of 20ng/mL of EGF and 20ng/mL of bFGF (both from R&D systems, Lille, France) and 5µg/ml of Heparin (Sigma Aldrich, St-Louis, USA) is conducted to enhance neuronal specification. Cells were fed every 3 days by changing half of the medium, and split every 5 days.

## 2.4 MIAMI CELL DIFFERENTIATION

### 2.4.1 MIAMI cell transfection

MIAMI E/F cells were seeded at 3000 cells per cm<sup>2</sup> coated with laminine (2µg/cm<sup>2</sup>, Sigma Aldrich, St-Louis, USA). Experiments were performed in MIAMI Opti-MEM<sup>®</sup> media (Life technologies, France). SiRNA-LNCs were incubated with cells at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub> for 4h before serum addition. Cells were harvested at appropriate times after culture and assayed for mRNA expression levels by RT-qPCR or protein expression by immunofluorescence.

### 2.4.2 LNC cell time retention

MIAMI cells were seeded on glass at 3000 cells per cm<sup>2</sup> coated with laminine (2µg/cm<sup>2</sup>, Sigma Aldrich, St-Louis, USA). Experiments were performed in MIAMI Opti-MEM<sup>®</sup> media (Life technologies, France). SiRNA fluorescent LNC and 100 n LysoTracker Red (Molecular Probes, Eugene, OR, U.S.A.) were incubated with cells at 37 °C in a humidified atmosphere with 5 %

CO<sub>2</sub> for 4h before phosphate buffered saline (PBS) washing and paraformaldehyde fixation or serum addition. After washing, cells were visualized from day 0 to day 6 post transfection using a fluorescence confocal multispectral imaging, FCSI (Leica TCS SP8, France).

### 2.4.3 MIAMI cell neuronal differentiation

MIAMI cells were seeded at 3000 cells per cm<sup>2</sup> coated with laminine (2µg/cm<sup>2</sup>, Sigma Aldrich, St-Louis, USA). Then, different conditions were tested to obtain the best GABAergic differentiation protocol (**Table 1**). Briefly, the differentiation was performed in two steps. In the first step, allow to obtain LGE: DMEM/F12 (Glutamax, Gibco, Life Technologies, Paisley, UK) was supplemented with N2 (both from Gibco, Life Technologies, Paisley, UK), and 200 ng/ml of Sonic hedgehog (SHH, Peprtech, Rocky Hill, USA) during fourteen days. In the second step, for GABAergic differentiation: Neurobasal media (Neurobasal, Gibco, Life Technologies, Paisley, UK) was supplemented with 10µM of valproic acid (Sigma Aldrich, St-Louis, USA) and 30ng/ml of BDNF (Peprtech, Rocky Hill, USA) during fourteen days or followed by BDNF treatment during 7 days. Length and surface area were quantified using MetaVue software<sup>®</sup>. 6 pictures from each condition (24 in total) were performed with X10 objective and used to determine total area and length. Only cells responding to the treatment (with neurite like structures) were evaluated in this experiment.

Media	DMEM-F12	Neurobasal	N2	B27	SHH	VPA	BDNF
<b>STEP 1:</b>							
Medium 1	X		15%		200ng/ml		
<b>STEP 2:</b>							
Medium 1: VPA + BDNF		X		2%		10 $\mu$ M	30ng/mL
Medium 2: VPA		X		2%		10 $\mu$ M	
Medium3: BDNF		X		2%			30ng/mL

**Table 1. Different media tested for the differentiation of MIAMI cells**

## 2.5 REVERSE TRANSCRIPTION AND REAL TIME QUANTITATIVE PCR

The following experiments were performed following the guidelines of the PACEM core facility ("Plate-forme d'Analyse Cellulaire et Moléculaire", Angers, France). Sense and antisense primer pairs (Eurofins MWG Operon, Ebersberg, Germany) were mixed in water at a final concentration of 5  $\mu$ M (**Table 2**). Total RNA of cells was extracted, purified using RNeasyMicrokit (Qiagen, Courtaboeuf, France), treated with DNase (10 U DNase I/ $\mu$ g total RNA) and its integrity verified on Experion RNA StdSens chip (Bio-Rad). RNA concentrations determined with a ND-2000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA) were used for normalization of the Reverse Transcription (RT). First strand complementary DNA (cDNA) synthesis was performed with a SuperScript<sup>TM</sup> II Reverse Transcriptase (Invitrogen), in combination with random hexamers, according to the manufacturer's instructions. cDNAs were then purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 40  $\mu$ L water (Gibco). 3ng of cDNA was mixed with Maxima<sup>TM</sup> SYBR Green qPCR Master Mix (Fermentas) and primer

mix (0.3  $\mu$ M) in a final volume of 10 $\mu$ L. Amplification was carried out on LightCycler 480 (Roche) with a first denaturation step at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 30 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. Two housekeeping genes, Glyceraldehyde-3- phosphate dehydrogenase (Gapdh), Beta actin (Actb), were tested for normalization. The GeNorm™ freeware (<http://medgen.ugent.be/~jvdesomp/genorm/>) was used to determine that GAPDH and ACTB were the two most stable housekeeping genes. The relative transcript quantity (Q) was determined by the delta Cq method  $Q = E^{(Cq \text{ min in all the samples tested} - Cq \text{ of the sample})}$ , where  $E = 2$  if the primer efficiency = 100%. It was normalized using the multiple normalization method described in Vandesompele et al (Vandesompele et al., 2002).  $Q \text{ normalized} = Q / (\text{geometric mean of the three most stable housekeeping genes } Q)$ . The  $2^{-\Delta\Delta Ct}$  method was retained, using housekeeping genes and gene of interest (Livak and Schmittgen, 2001) tested on control sample and treated sample.

Gene	Full name	NM accession number	sequences
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase	<a href="#">NM_001289745.1</a>	Fwd: CAAAAGGGTCATCATCTCTGC Rev: AGTTGTCATGGATGACCTTGG
<b>ACTB</b>	Actin	<a href="#">NM_001101.3</a>	Fwd: CCAGATCATGTTTGAGACCT Rev: GGCATACCCCTCGTAGAT
<b><math>\beta</math>3-TUB</b>	Tubulin beta 3 class III	<a href="#">NM_006086</a>	Fwd: CCAGTATGAGGGAGATCG Rev: CACGTACTIONTGTGAGAAGAGG
<b>REST</b>	RE1-silencing transcription factor	<a href="#">NM_001193508.1</a>	Fwd: ACTCATACAGGAGAACGCC Rev: GTGAACCTGTCTTGCATGG
<b>SCG10</b>	stathmin 2	<a href="#">NM_001199214.1</a>	Fwd: TGTCACTGATCTGCTCTTGC Rev: AGAAGCTAAAGTTTCGTGGGG
<b>NFM</b>	neurofilament, medium polypeptide (Variant 1)	<a href="#">NM_005382.2</a>	Fwd: GACCTCAGCAGCTACCAG Rev: TAGTCTCTTACCCTCCAG
<b>BDNF</b>	Brain-derived neurotrophic factor	<a href="#">NM_001143816</a>	Fwd: CAAACATCCGAGGACAAGG Rev: TACTGAGCATCACCTGG
<b>TrkB</b>	Tropomyosin receptor kinase B	<a href="#">NM_006180</a>	Fwd: TTGTCTGAACTGATCCTGGTGGGC Rev: AGGTTAGGTGCGGCCAGATTTGC
<b>GAD67</b>	Glutamic Acid Decarboxylase 67	<a href="#">NM_000817</a>	Fwd: GGTGGCTCCAAAATCAAAGC Rev: CAATGTCAGACTGGGTAGCG
<b>DARPP32</b>	Dopamine- and cAMP-regulated phosphoprotein,	<a href="#">NM_181505</a>	Fwd: GAGAGCCTCAGGAGAGGG Rev: CTCATTCAAATTGCTGATAGACTGC

<b>Pax6</b>	Mr 32 kDa		
	Paired box 6	<a href="#">NM_000280</a>	Fwd: TTTCAGCACCAAGTGTCTACC Rev: TAGGTATCATAACTCCGCC
<b>Oct3/4</b>	Octamer-binding transcription factor 4	<a href="#">NM_203289</a>	Fwd: GGAAGGTATTCAGCCAAACG Rev: GTTCGCTTCTCTTTCCGGG
	Homeobox Transcription Factor Nanog	<a href="#">NM_024865</a>	Fwd: GATCCAGCTTGTCCCCAAAG Rev: GCTGTCTGAATAAGCAGATCC
<b>Col2A</b>	Collagen type II, alpha 1	<a href="#">NM_001844</a>	Fwd: GAGGGGATCGTGGTGACAAAGG Rev: TTGCATTACTCCCAACTGGGCG
	Fatty acid binding protein 4, adipocyte	<a href="#">NM_001442</a>	Fwd: ACAGCACCTCCTGAAAAGTGC Rev: TGTTAGGTTTGGCCATGCCAGC
<b>Runx2</b>	Runt related transcription factor2	<a href="#">NM_001015051</a>	Fwd: ACAAATCCTCCCCAAGTAGC Rev: GACACCTACTCTCATACTGGG
	Distal-less homeobox 2	<a href="#">NM_004405</a>	Fwd: GACCTTGAGCCTGAAATTCG Rev: ACCTGAGTCTGGGTGAGG
<b>VEGFA</b>	Vascular endothelial growth factor A	<a href="#">NM_001204384</a>	Fwd: CAGCGCAGCTACTGCCATCCA Rev: CAGTGGGCACACACTCCAGGC
	Nerve growth factor	<a href="#">NM_002506</a>	Qiagen, ref #QT00043330
<b>GDNF</b>	Glial cell line-derived neurotrophic factor	<a href="#">NM_01167.2</a>	Qiagen, ref #QT00001589

**Table 2. Sequence of primers validated in RT-qPCR**

## 2.6 IMMUNOFLUORESCENCE

After treatment, cells were fixed by addition of 1ml of ice cold 4% paraformaldehyde (PFA, Sigma, St Louis, USA) in PBS (Lonza, Verviers, Belgium) pH 7.4 during 15min. Then cells were washed in DPBS three times. Non-specific sites were blocked with DPBS, Triton 0.1% (PBS-T, Triton X-100, Sigma, St Louis, USA), bovine serum albumin 4% (BSA, Fraction V, PAA Lab, Austria), normal goat serum 10% (NGS, Sigma, St Louis, USA) during 45 min at RT. A mouse anti human  $\beta$ 3-tubulin (2ng/ml, clone SDL.3D10, Sigma, St Louis, USA), a mouse anti human neurofilament medium (NFM, 1:50, clone NN18, Sigma Aldrich, USA), a monoclonal rabbit anti human dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32, 1:400, clone EP721Y, Abcam, Paris, France), A mouse anti glutamic acid decarboxylase-67 antibody (GAD67, 5 $\mu$ g/ml, clone 1G10.2, Millipore SA, Guyancourt, France), were used to characterize cell differentiation. Cells were incubated overnight with the primary antibody diluted in PBS-T, BSA 4% at 4°C. After washes, slices were

incubated with the biotinylated mouse or rabbit secondary antibody (7,5 µg/ml, Vector Laboratories, Burlingame, USA) for 1 hour at RT. Then slices were washed and incubated with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS for 1 hour before mounting with a fluorescent mounting medium. They were observed with a fluorescence microscope (Axioscop, Carl Zeiss, LePecq, France).

## 2.7 PHARMACOLOGICALLY ACTIVE MICROSPHERES

### 2.7.1 BDNF microspheres preparation

PLGA-P188-PLGA, which was synthesized by IBMM-CRBA CNRS UMR 5247 (Montpellier, France). BDNF Microspheres were prepared using a solid/oil/water (s/o/w) emulsion solvent evaporation–extraction process previously described [39–41]. Protein loading was 1 µg of protein and 5 µg of human serum albumin (HSA)/mg of MS. BDNF and HSA were nano-precipitated separately using a process previously described [39] but adapted to lyophilized BDNF. Briefly the protein powder was first dissolved in a non-buffered aqueous solution of sodium chloride containing poloxamer and this solution was introduced into glycofurol. After 30 min at 4 °C, the nanoprecipitated proteins were recovered by centrifugation and dispersed in the organic phase (670 µL of 50 mg PLGA–P188–PLGA dissolved in a 3:1 methylene chloride:acetone solution). The suspension was then emulsified in a poly(vinyl alcohol) aqueous solution (30 mL, 6% w/v at 1 °C) and mechanically stirred at 995 rpm for 1 min. After addition of 33 mL of deionized water and stirring for 10 min, the emulsion was added to 167 mL deionized water and stirred for 20 min to extract the organic solvent. Finally, the MS were filtered on a 5 µm High Volume Low Pressure (HVLP) type filter, washed and freeze-dried. MS without protein were prepared following the same process, and called blank-MS or blank-PAMs when covered with laminin.

### 2.7.2 LM-PAM preparation & characterization

PLGA-P188-PLGA MS were coated with LM and poly-D-Lysine (PDL). Coating solutions were prepared in Dulbecco's Phosphate-Buffered Saline DPBS. The concentration of the coating molecules was 6 µg/mL of LM and 9 µg/mL of PDL (corresponding to a 40:60 ratio of LM:PDL). 5 mg of MS was

suspended in DPBS and sonicated until full dispersion of the MS. The solution containing PDL and LM molecules was mixed to the MS suspension (final volume: 10 mL) and placed under rotation at 15 rpm at 37 °C during 1h30min. After coating, LM-PAMs were washed 3 times in sterile distilled water containing 1% antibiotic, lyophilized and kept at -20 °C. Each tube was covered with sigmacote® to prevent product loss on the tube walls. The laminine surface was characterized by confocal microscopy after LM immunostaining. Lyophilized PAMs (1 mg) were suspended in DPBS containing 4% bovine serum albumin (BSA), 0.2% Tween 20 and incubated for 30 min at room temperature (Rt) under 15 rpm stirring. Samples were then washed three times with DPBS and centrifuged (9000 g, 5 min). Anti-LM mouse monoclonal antibody (100 µg/mL in DPBSBT) was incubated at 37 °C for 1.5 h under rotation. Samples were then washed 4 times before incubation with biotinylated anti-mouse IgG antibody (2.5 µg/mL in DPBS) for 1 h, at RT, under rotation. After three washes, samples were incubated with streptavidin-fluoroprobe 547 (1:500 in DPBS) at RT, for 40 min, under rotation. Samples were observed under confocal microscopy (Olympus Fluoview™ TU 300, Rungis, France). Three independent experiments were performed and every condition was observed in triplicate.

### 2.7.3 Formation of PAM-cell complexes

MIAMI differentiated cells were detached and pelleted at 295g for 10 min. Pellets were resuspended in culture medium supplemented with 3% FBS (Lonza, Verviers, Belgium). Lyophilized microspheres (0.50mg) were resuspended in coated eppendorf tubes (Sigmacote, Sigma, St Louis, USA) containing DMEM-F12 (Gibco, Life Technologies, Paisley, UK), for 15 min. PAM suspension was mixed with 0.5mL of cell suspension ( $2.5 \times 10^5$  cells/0.50 mg PAMs). The mixture was then gently flushed and plated in 1.9 cm<sup>2</sup> Costar ultra-low adherence plate (Corning, Avon, France). Plates were incubated at 37°C during 4h for to allow cell attachment on PAM surface. PAMs/cell aggregates were pelleted by centrifugation at 200g for 2 min. Cell adhesion to PAM surface was assessed by microscopic observation and cells adhered to PAMs were quantified using the Cyquant cell proliferation assay (CyQuant Cell proliferation Assay kit, Invitrogen). Complexes were further studied using light and fluorescence

microscopy and scanning electron microscopy. Samples were prepared for scanning electron microscopy analysis as previously described [34].

## 2.8 INJECTION OF PAMS-CELLS IN ORGANOTYPIC SLICES

### 2.8.1 Organotypic slices preparation

Six to eight days Sprague Dawley rat pups were sacrificed after anaesthetic and brains were removed. Cerebral hemispheres were separated and glued on the vibratome plate on their central side. 400µm coronal slices were obtained using a vibratome (motorized vibroslice, Campden instruments, Loughborough, England) °. Cortico-striatal slices were then dived into Grey's Salt Balanced Solution (Sigma Aldrich) supplemented with 6,5mg/L of glucose (Sigma Aldrich, St-Louis, USA) and antibiotics (Sigma Aldrich, St Louis, USA). Three to four slices per hemisphere were next transferred to 30 mm diameter semi-porous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and put in culture at 37°C, 5% CO<sub>2</sub>. From day 0 to day 3, a serum containing medium was used: 50% MEM (Minimum Essential Medium Eagle, Sigma Aldrich), 25% Hank's (Hank's Balanced Salt Solution, Sigma Aldrich), 25% of horse serum (decomplemented horse serum, Gibco), 6.5mg/ml of glucose, 1 mM of L-glutamine (Sigma Aldrich, St-Louis, USA) and antibiotics (Sigma Aldrich, St-Louis, USA). From day 3 to day 16, a serum free medium was used: neurobasal medium (Gibco) supplemented with 6.5mg/L of glucose, 1mM of -glutamine (Sigma Aldrich, St Louis, USA), B27 supplements (50x, B27 supplements, Gibco) and antibiotics (Sigma Aldrich, St-Louis, USA) this media was changed every two days.

### 2.8.2 Injection of stem cell/ PAM complexes into organotypic slices

Five days after organotypic slice preparation, treatments were injected into the striatum using a 22-gauge needle (Hamilton, Bonaduz, Switzerland) connected to a micromanipulator. Three experimental groups were tested: (1) MIAMI siREST SHH cells, (2) MIAMI siREST SHH LM-PAM, and (3) MIAMI siREST SHH LM-PAM-BDNF. Total injection volume consisted of 4µl of culture media containing approximately 100.000 cells alone or adhered to 0.2 mg of PAMs. Injections were done at 0.5µl/minute infusion rate. The needle was

left in place for 5 min to avoid the cells being expelled from the organotypic slices.

### 2.8.3 Histological study

Seven days after injection, organotypic slices were fixed by addition of 5ml of ice cold 4% PFA (Sigma, St Louis, USA) in PBS (Lonza, Verviers, Belgium) pH 7.4 during 2 hours. Then slices were washed in PBS three times. Nonspecific sites were blocked with PBS, Triton 1% (PBS-T, Triton X-100, Sigma, St Louis, USA), BSA 4% (Fraction V, PAA Lab, Austria), NGS 10% (Sigma, St Louis, USA) during 4 hours at RT under agitation (except for CD31 study, in this case, 0.05% Triton is employed).

MIAMI cells were detected using a mouse anti-human mitochondria antibody (10ng/ml, clone MTCO2, Abcam, Paris, France). A monoclonal rabbit anti human dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32, 1:400, clone EP721Y, Abcam, Paris, France), antibody was used to characterize grafted cells differentiation. Slices were incubated 48h with the primary antibody diluted in PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the corresponding biotinylated mouse or biotinylated rabbit secondary antibody (7.5 µg/ml, Vector Laboratories, Burlingame, USA). Then slices were washed and incubated with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS for 2 hours before mounting with a fluorescent mounting medium. Isotypic controls and/or omission of the primary antibody were performed to assess the specificity of the immunostainings. Slices were observed with a fluorescence microscope (Axioscop, Carl Zeiss, LePecq, France).

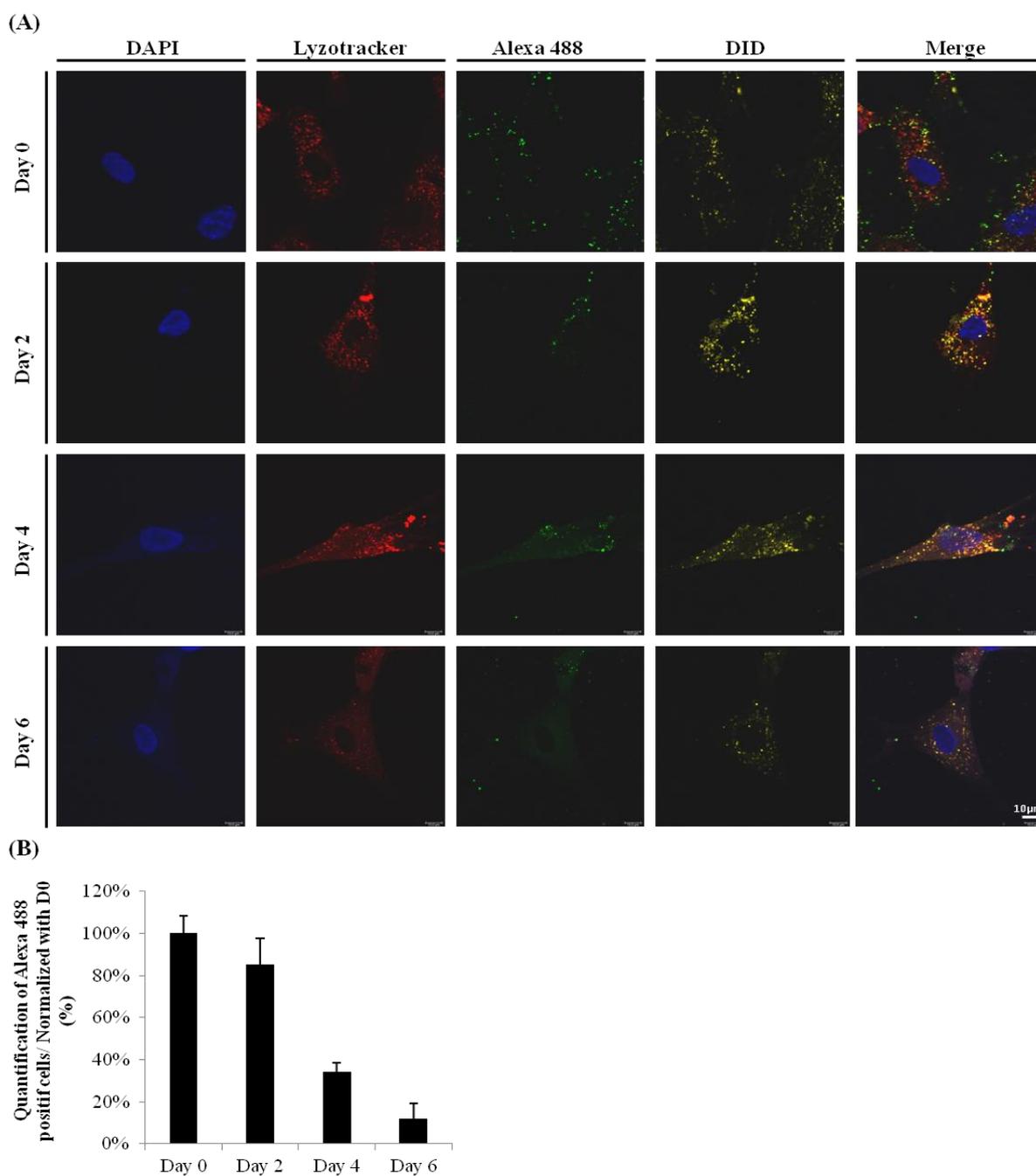
## **3. RESULTS**

### **3.1 CELLULAR UPTAKE AND LNC TIME RETENTION IN MIAMI CELLS**

SiRNA delivery and cell time retention of LNCs was studied on MIAMI E/F cells by fluorescence confocal multispectral imaging, FCSI (**Figure 1A**). In order to follow both the LNC and the siRNA distribution, double-labelled LNCs were generated by loading them with the DiD and with the siRNA-Alexa488.

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Lysosomes were observed with the lyzotracker. Directly, after transfection, named Day 0, we observed a varying number of LNCs in the majority of MIAMI E/F cells **(Figure 1A and 1B)**. The heterogeneity of LNC distribution in each cell disappeared at Day 2. **(Figure 1A)**. The number of positive cells for the siRNA decreased progressively but we still observed 12% of positive cells at day 6 **(Figure 1B)**. In general, there are more DiD tagged LNCs in the cells than fluorescent siRNA as indeed the encapsulation efficiency of siRNA-REST in LNC's is around 50%. Although many LNCs co-localized with lysosomes, we clearly observed that the siRNA did not co-localize, suggesting that it was released.

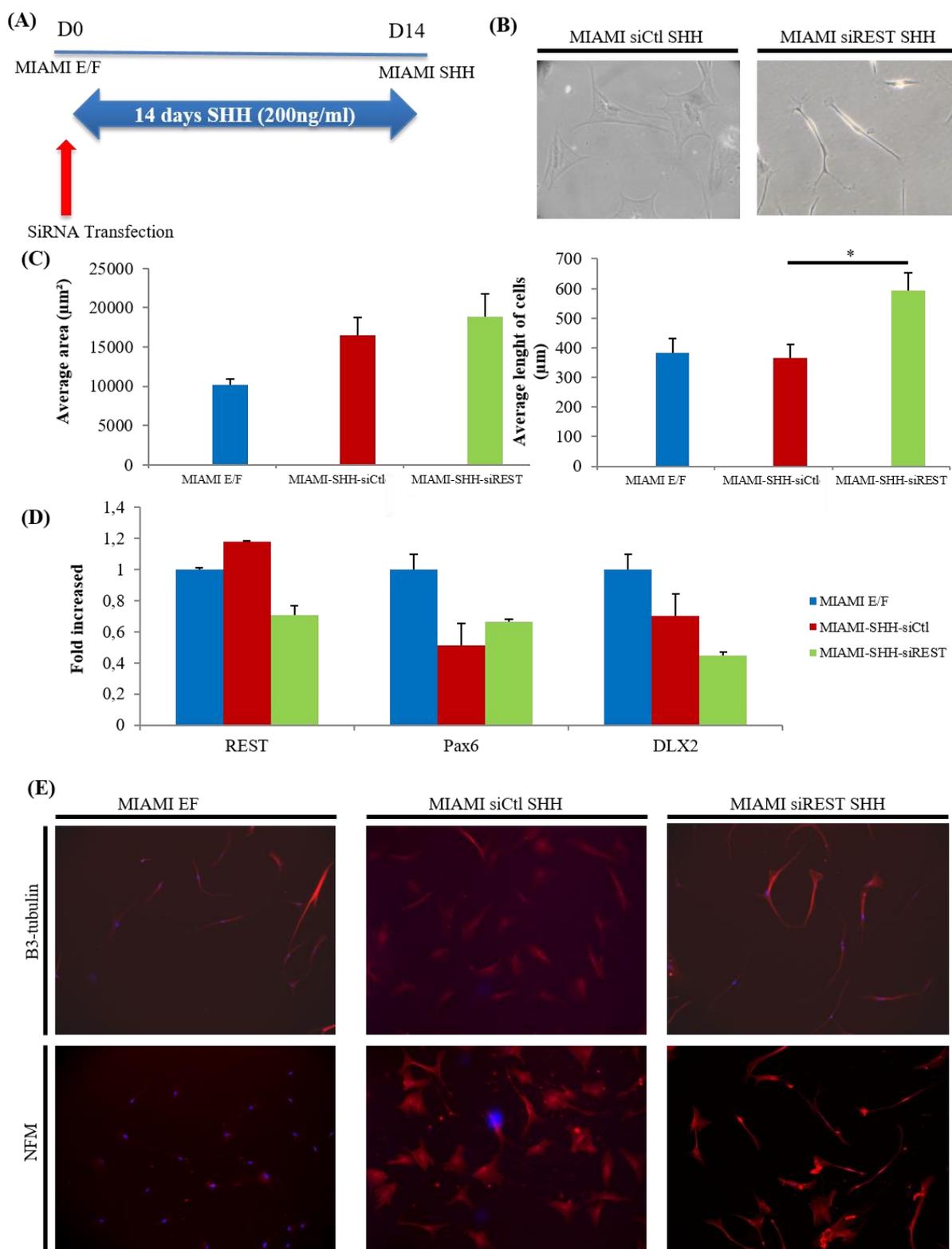


**Figure 1. Cellular uptake and retention time of siRNA-LNC in MIAMI E/F cells.** After 4 hours' incubation, confocal microscopy was performed on MIAMI cells with siRNA LNCs. Cells were fixed on glass slide and nucleus staining was performed with DAPI (blue). Double fluorescent probes were used to follow siRNA LNCs: lipophilic DiD (yellow) and Alexa488 siRNA (green). Analysis confirmed the internalization of siRNA LNCs and its presence until day 6 (A) Positive siRNA cells were counted using ImageJ. 6 images per condition in X10 objective were selected (B) Scale bar represents 10µm.

## 3.2 MIAMI CELL NEURONAL COMMITMENT

We previously described MIAMI E/F as neural precursors [24]. In order to obtain neuronal precursors of the lateral ganglionic eminence like (LGE-like) that later give rise to the striatal GABAergic neurons, we used SHH as an inductor (**Figure 2A**). To investigate the role of REST silencing to better induce the neuronal commitment of MIAMI E/F, we transfected siREST and a siRNA control, named siCtl. Fourteen days after treatment, most of the MIAMI-siREST SHH cells exhibited a neuron-like morphological change, with long neurite-like structures (**Figure 2B**). The density of cells during the first step is very important and if the confluence is superior to 30%, over-proliferation is observed (data not shown). No obvious morphological changes were observed in MIAMI-siCtl-SHH under these same culture conditions. There was a tendency to increase the cell surface (**Figure 2C**), and the majority of cells presented a total length of around 600 $\mu$ m (**Figure 2C**) due to the long neurite-like structures. Thirty percent inhibition of mRNA of REST was still observed fourteen days after transfection compared with siCtl expression (**Figure 2D**).

LGE progenitors express Gsx2, Dlx2, and Meis2 and to a lesser degree Pax6. To further characterize these cells, we performed RT-PCR analysis to detect the decrease of embryonic markers and the increase of neural/neuronal differentiation markers. The expression of pluripotent markers has been quantified and some variations on results are observed (**supplementary data**). The expression of Pax6 involved during the neurogenesis decreased slightly fourteen days after the treatment as described in the literature (**Figure 2D**). Dlx2, a major gene, characteristic from LGE progenitors is strongly expressed in MIAMI E/F cells and decreased at the end of the commitment (**Figure 2D**).  $\beta$ 3-tubulin protein increased during the differentiation with siREST further suggesting obtaining LGE-like progenitors (**Figure 2E**). Furthermore, the immunofluorescence of neurofilament (NFM) protein revealed a high expression in MIAMI-siREST-SHH, which was not detected in MIAMI E/F cells (**Figure 2E**).



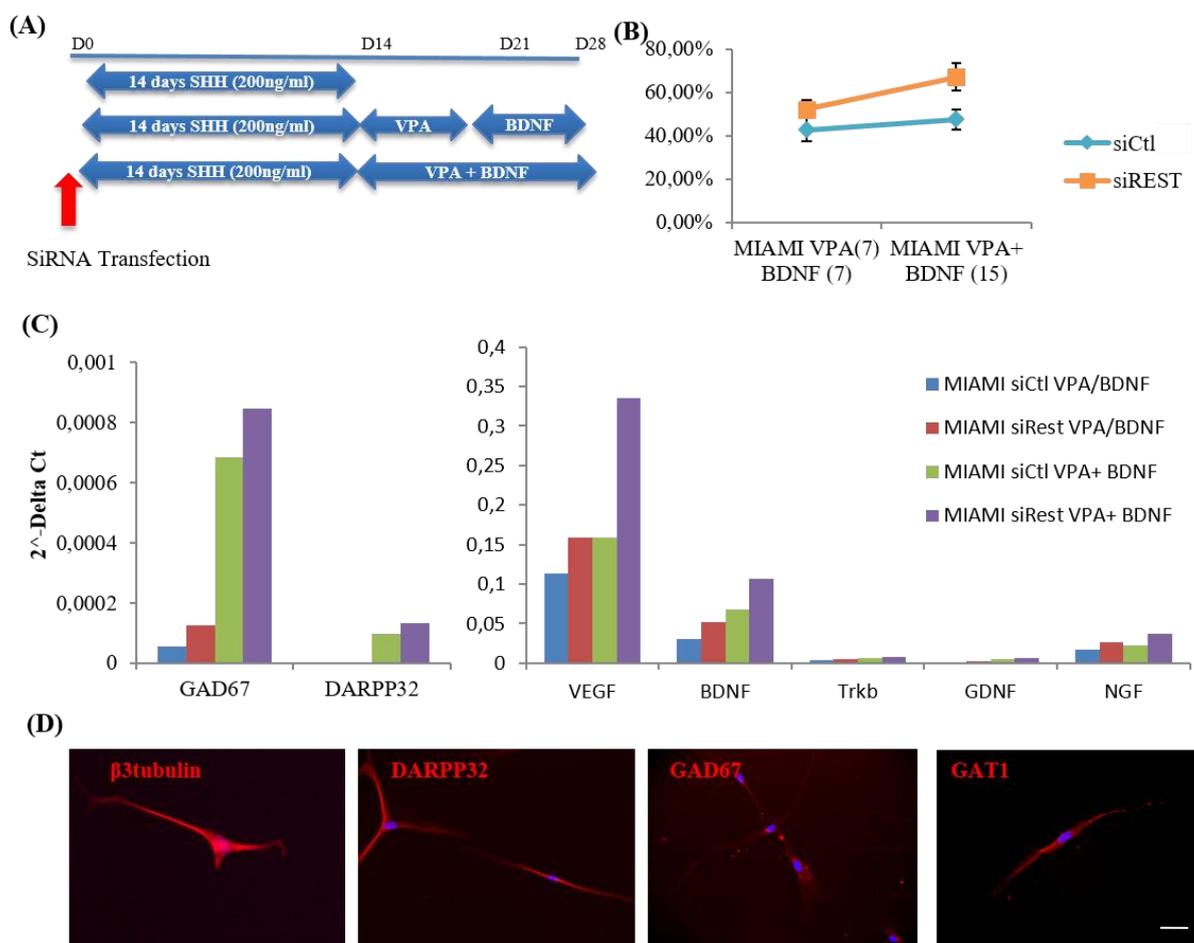
**Figure 2. Specification of MIAMI siREST committed into LGE-like progenitors.** For neuronal commitment, a simple protocol has been designed using sonic Hedgehog protein. Transfection of MIAMI E/F was performed with 250 ng/ml of siControl and siREST-LNCs (A). During the culture period of 14 days, MIAMI-siCtl-SHH cells show very few morphological changes. In contrast, MIAMI-siREST-SHH stretched out short neurite at 7 days, while at 14 days later, almost all of the MIAMI-siREST-SHH exhibited a neuron-like morphology (B). The quantification of surface area and the measure of cell length was performed fourteen days after transfection for MIAMI E/F, MIAMI-siCtl-SHH and MIAMI-siREST-SHH (C) the characterization of the differentiation was realized by RT-qPCR and immunofluorescence. The expression of genes

REST, Pax6, Dlx2 (D), was quantified fourteen days after the commitment. Results were expressed in Fold increase. Results (n=3) are expressed as mean measure  $\pm$  standard deviation. In vitro immunofluorescence against  $\beta$ 3-tubulin and NFM on MIAMI E/F, MIAMI-siCtl-SHH and MIAMI-siREST-SHH (F) Scale bar = 50  $\mu$ m.

### 3.3 MIAMI CELL DIFFERENTIATION TO A GABAERGIC LIKE PHENOTYPE

The LGE-like progenitors were further exposed to Valproic acid (VPA) for 7 days and then BDNF until day 28 (named VPA/BDNF) or to a mix of VPA and BDNF for 15 days (named VPA+BDNF) (**Figure 3A**). At this step, no further morphological change was observed. However, siREST increased the number of cells responding to the second protocol using VPA (10 $\mu$ M) and BDNF (30ng/mL) in the same time treatment (**Figure 3B**). In other words, 45,09%  $\pm$  3,4 of cells presented neurite-like structures with siCtl and 59,63%  $\pm$  10,4 with siREST. At the end of the differentiation, only a slight expression of Dlx2 or REST was detected by RT-qPCR (**Supplementary data**). A high expression of  $\beta$ 3-tubulin was observed in all conditions. Importantly, the majority of the GABA-like cells were positive for dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP32) and glutamate decarboxylase (67kDa), a feature of MSNs. These two proteins appeared when VPA and BDNF were in combination (**Figure 3C**). With siREST and VPA/BDNF;  $\beta$ 3-tubulin, GAD67, DARPP32 and GAT1 protein were detected (**Figure 3D**) suggesting a GABAergic like differentiation enhanced by siREST.

MIAMI E/F cells are very interesting because they secrete many tissue repair factors [24]. The expression of mRNA *in vitro* allowed detecting which factors may be secreted by cells alone. In all cases, VEGFa, BDNF and NGF were expressed demonstrating the potential neuroprotective effect of MIAMI differentiated cells in the HD (**Figure 3C**). Nonetheless, the mRNA of those factors were highly present in MIAMI-siREST-VPA+BDNF when compared with the other conditions; two-fold higher than MIAMI-siREST-VPA/BDNF. These results need to be confirmed.



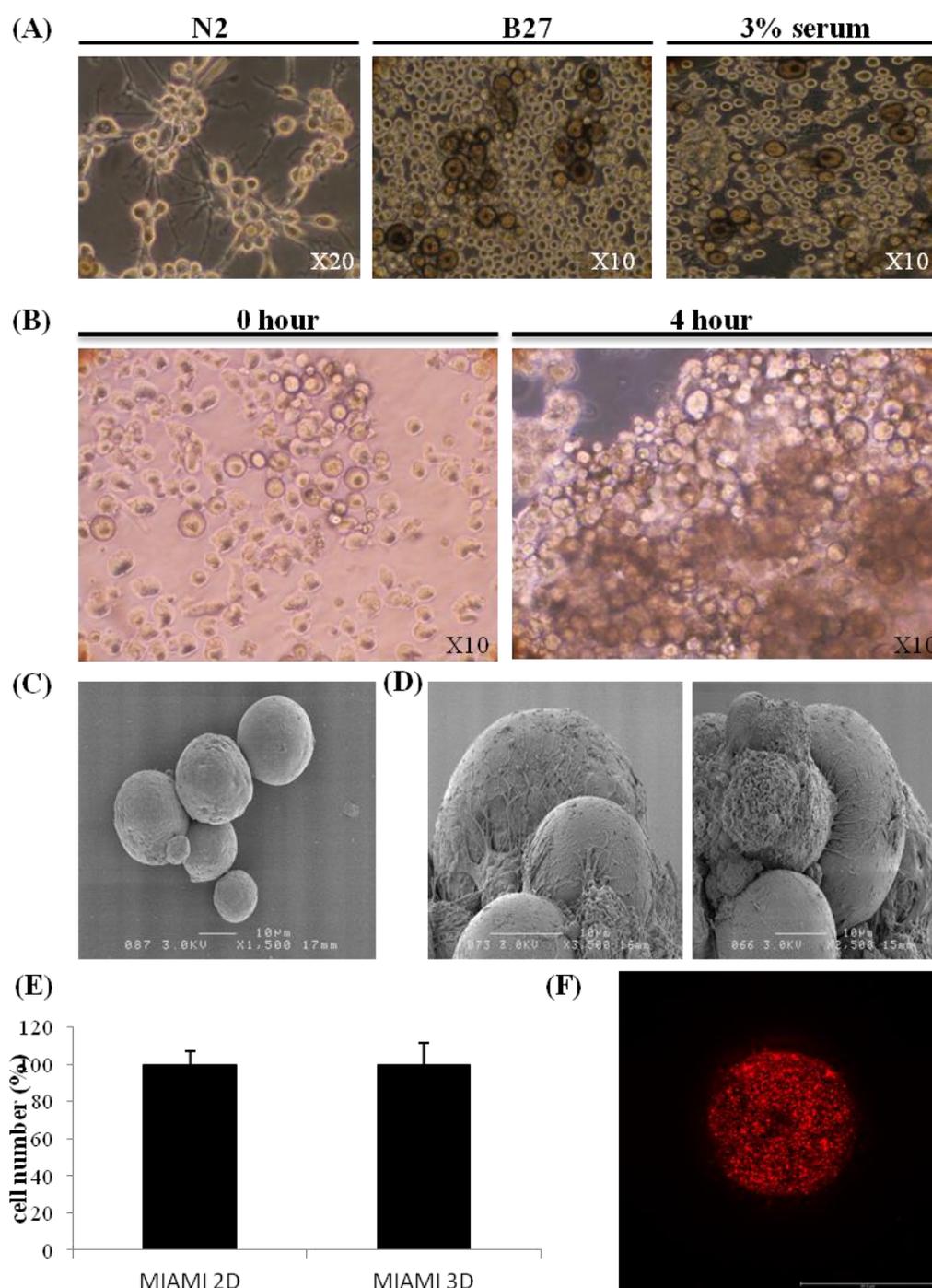
**Figure 3. Differentiation and Characterization of GABA neurons.** Schematic procedure of GABAergic differentiation (A). The quantification of cell length superior to 600µm considered as positive response to the treatment, was performed fourteen days after the differentiation for MIAMI-siCtl-VPA/BDNF, MIAMI-siRest-VPA/BDNF, MIAMI-siCtl-VPA+BDNF, and MIAMI-siREST-VPA+BDNF (B). The characterization of the differentiation was realized by RT-qPCR and immunofluorescence. The expression of genes *Dlx2*, *REST*,  $\beta$ 3 Tubulin, *DARPP32* and *GAD67* (C) was quantified at the end of the differentiation Results (n=1). Cytokines expression profile of differentiated cells was also determined by RTqPCR of cells alone at the end of the differentiation (C). For both cells *VEGFA* mRNA is the most expressed followed by *BDNF* mRNA and *NGF*. Results were in  $2^{-\Delta\Delta Ct}$ . Results (n=1). In vitro immunofluorescence against  $\beta$ 3-tubulin, *DARPP32*, *GAD67*, *GAT1* on MIAMI-siREST-VPA+BDNF (D) was performed. Scale bar = 50 µm.

### 3.4 CHARACTERIZATION OF PAM/CELLS COMPLEXES

The particle size of PAMs measured using a Multisizer Coulter Counter was around 30µm (data not shown). Observation of the microspheres and PAMs by bright field microscopy and with scanning electron microscope was

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performed to ensure the quality of the formulation **(Figure 4A and 4C)**. Microspheres were perfectly spherical with a smooth surface, and no pores on their surface **(Figure 4C)**. During the commitment and the differentiation, no serum and no antibiotics were used. In order to respect these conditions different media for cell adherence were tested with MIAMI SHH cells **(Figure 4A)**. Unfortunately, we observed a high proportion of cells adhered to the plastic or cells alone with B27 and N2 media, which is not the case for 3% of serum **(Figure 4A)**. PAMs with LM surface allowed MIAMI SHH cells adherence on their surface in 4hours **(Figure 4B and 4D)**. The percentage of cells adhered onto PAMs' surface at the end of the cell attachment protocol was about 95% **(Figure 4E)**. The optimal attachment could be explained by the homogenous coating of laminin observed with confocal microscopy **(Figure 4F)**.

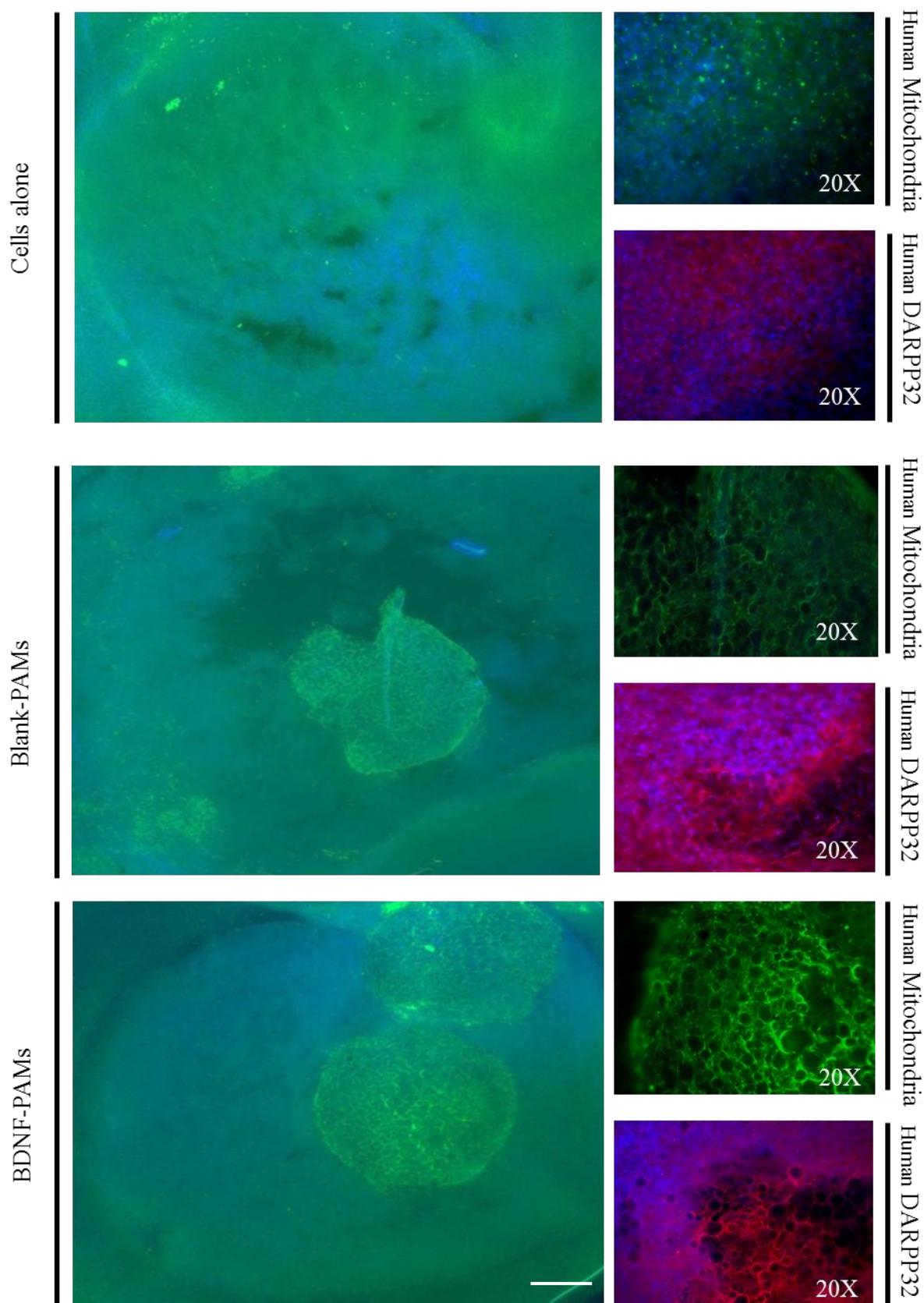


**Figure 4.** Adherence of MIAMI siRNA-SHH on PAMs. Different media for adherence were tested (A). We selected the media with 3% of serum for the rest of experiments. In this condition, we observe the adherence onto PAMs by brightfield microscopy at time 0 and 4H after cells and PAMs contact (B). Observation of blank PAMs (C) and cells/PAMs complexed (D) by scanning electronic microscopy. Cells number after 4h adherence onto PAMs was quantified by Cyquant® Reagent (E). Immunofluorescence of laminin on PAM was observed by confocal microscopy (F).

### 3.5 INJECTION OF PAM/CELLS IN ORGANOTYPIC SLICES HD

Cells (MIAMI siREST-SHH) alone or complexed to Blank-PAMs or BDNF-PAMs were grafted in organotypic slices at day 5 which represent 30% of GABAergic striatal cell degeneration. Immunofluorescence against human Mitochondria was used to visualize MIAMI cells in rat brain, 7 days after grafting (**Figure 5**). PAMs clearly improved survival of cells. Indeed, immunofluorescent staining was faint with cells alone, suggesting that some cells died. Blank-PAMs improved the survival compared to cells alone but the intensity of fluorescence was higher with BDNF-PAMs (**Figure 5**).

Immunofluorescence against DARPP32 was used to determine MSN-like differentiated MIAMI cells 7 days after grafting in the HD organotypic slices. Very few cells were positive for DARPP32 and only with BDNF-PAMs was there some immunofluorescence clearly different from the background staining, suggesting the expression by MIAMI cells of DARPP32 with this condition (**Figure 5**). Nonetheless, these observations have been realized only seven days after grafting, and the mRNA of DARPP32 appeared fourteen days after treatment in vitro.



**Figure 5. Ex vivo GABAergic MSN differentiation of grafted cells.** Immunofluorescence against Human mitochondria and human DARPP32 for MIAMI cells alone, seven days after grafting of cells or complexes with blank or BDNF PAMs. Scale bar is 150 $\mu$ m.

## 4. DISCUSSION

The combination of mesenchymal stem cells and polymeric bioactive scaffold to enhance cell survival and differentiation needs to be developed to improve therapeutic approaches for HD. In this study we showed that a subpopulation of MSCs, the MIAMI cells were able to differentiate towards the neuronal GABAergic lineage by an epigenetic ARN interfering approach inhibiting REST expression combined to GABAergic inducers. Moreover, these cells committed towards this phenotype cells could be transplanted with PAMs delivering BDNF in an *ex vivo* model of HD, survive and maintain this GABAergic neuronal precursor phenotype, particularly in response to BDNF.

It has been shown that transplantation of MSCs into QA model of HD led to improvement in behavior and reduced the lesion volume. These beneficial effects could be explained by the secretion of neurotrophic factors including BDNF, CNTF, nerve growth factor, insulin-like growth factor 1 and epidermal growth factor [42]. Within this line, previous studies have shown that MIAMI cells release more tissue repair factors than MSCs [22]. Moreover, in a PD model and after stroke their tissue repair capacity allowed neuroprotection of these damaged cells [14,34,35]. Indeed, in this study MIAMI cells conserved their paracrine effect even at the end of the differentiation with high level of VEGFA, BDNF and NGF mRNA. Although, it was previously reported that the addition of GDNF on organotypic slices cultures media enhanced the expression of DARPP32 in striatal neurons [30], the low level of GDNF should not increase by itself DARPP32 in our model. However, the paracrine effect may be indirect and increase the survival not only of the transplanted cells but also of the issue parenchyma by an angiogenic effect. Indeed, it has been previously shown in an *ex vivo* model of PD that release of VEGF by MIAMI cells increased the vascularization around the graft site probably participating to the neuroprotective effects observed in this paradigm [35]. Secretion of neurotrophic factors have not been reported for ESC and iPS cells, which show the advantage of MIAMI cells for a neuroprotective treatment of HD. The release of these factors by the MIAMI cells alone or combined to PAMs needs to be further investigated. However, the lacks of consistent neuronal differentiation of transplanted MSCs have limited their therapeutic efficacy in slowing the

progression of HD-like symptoms in animal models of HD. Indeed, their capacity to differentiate into neuronal like cells is still insufficient.

MIAMI cells have already demonstrated their capacity to differentiate into dopaminergic neurons, with electrophysiological properties of immature neurons [14,24]. Based on the literature, we developed and tested a simple protocol with different steps in order to obtain GABA-like neurons [11–13]. During the first step we decided to inhibit REST and drive the commitment with sonic hedgehog. Indeed, we already demonstrated that REST inhibition induced spontaneously neural differentiation, increasing the mRNA of neurofilament (NFM). In particular, LNC siREST had a better effect on the differentiation than a commercial reagent (lipofectamine<sup>®</sup>) with siREST (paper in press). In order to better comprehend this effect, we followed LNC and siRNA retention time in MIAMI E/F cells during 6 days. The internalization of LNC seems to be by endocytosis because we observed concentrated spots exactly as those observed after endocytosis phenomenon such as with calcium phosphate particles recently developed [43]. More interestingly, LNC properties proved their capacity to destabilized the lysosomal compartment [21]. In this study, LNCs co-localized with lysosomes while this was not the case for the siRNA. The observation in confocal microscopy showed a high proportion of siRNA- positive cells during two days, and a progressive decrease of positive cells until day 6 where we found 15% of cells still presenting siRNA. Conversely, *in vitro* cell time retention of siRNA with lipofectamine and liposomes showed that the siRNA was present during only three days [44]. The progressive siRNA delivery in our study suggests that this LNC property is quite interesting for maintaining a differentiated cellular state for cell therapy applications.

The effect of siREST on the neuronal commitment to obtain lateral ganglionic eminence was promising. Through 14 days of differentiation of MIAMI E/F-siREST under commitment culture conditions [12], these cells gradually stretched out long neurite-like structures. The morphological similarity of these cells with neurons suggests that REST knock-down allows MIAMI cells to engage into the neuronal lineage. Furthermore, the cells presented some similarities to LGE precursor cells with the decrease of Pax6 (neural stem cell marker), the slight increase of  $\beta$ 3-tubulin (neuronal precursor

marker) and high expression of NFM (immature and mature neuron marker). However, the expression of *Dlx2*, a marker of GABAergic progenitors [45], was more important in MIAMI E/F or with the siCtl and the high expression of NFM suggests that the cells could be further committed in the GABAergic differentiation lineage. At the end of the differentiation, the knock-down of REST seemed to have a limited influence on the appearance of DARPP32 markers or GAD67 (mRNA and proteins). However, it led to an increased response to the best treatment, which was the combination of VPA with BDNF during the last stage of the differentiation protocol.

Pre-clinical trials with mesenchymal stem cell transplantation suggest that cell therapy is a potential promising option for HD [16,46]. Unfortunately, transplanted cells have low survival rate. In this study, we used carriers providing a biomimetic support of laminin and the delivery of BDNF, the PAMs, combined with MIAMI cells to maximize the resulting protective/repairative effects on HD model. This effect should be double, drive the MIAMI cell survival and differentiation as well as increase the neuroprotective effect that we hope to observe with LGE-like cells obtained from MIAMI-siREST committed cells. Indeed, the secretion of BDNF by MIAMI cells and the release by PAMs should have a potent neuroprotective action. As reported earlier, PAMs may improve the integration of PC12 cells and embryonic dopaminergic cells within the brain parenchyma, after transplantation in hemi-parkinsonian rats, by improving their survival and differentiation. This work clearly confirmed previous studies. Cells alone or complexed to LM-PAMs were grafted at day 5, when 30% of degeneration was obtained. A high detection of human Mitochondria was observed when the cells were complexed to BDNF-PAMs, which suggests that scaffold and BDNF improved the survival. More interestingly, slight expression of DARPP32, demonstrated their potential to differentiate into MSN in only seven days, probably driven by the released BDNF. In order to confirm that, secretome analyses with cells complexed to Blank or BDNF-PAMs should be performed. In any case, these data provide encouraging results arguing for an enhancement of the neuronal differentiation of MIAMI cells towards a GABAergic phenotype and a neuroprotective effect for HD with this innovative nano and micromedicine safe combinatorial strategy.

## CONCLUSION

In this study, we tested an innovative strategy combining stem cells and biomaterials in *ex vivo* model of HD. We demonstrated in this way, the capacity to our model to screen new therapy. We proved, *in vitro*, the capacity to MIAMI cells to differentiate into GABAergic-like neurons by REST inhibition and appropriate media. After graft, the survival of MIAMI cells combined with LM-BDNF-PAMs seems to be increased, probably due to the synergic effect between the 3D support and the release of BDNF. Nonetheless, the biological effect of committed MIAMI-LM-BDNF-PAMs on the HD environment needs to be characterized and understood.

## ACKNOWLEDGMENTS

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**Supplementary data**

Markers Normalized with MIAMI E/F	LGE commitment	Fold increased (RT-qPCR)	GABA differentiation	Fold increased (RT-qPCR)
Pluripotent markers				
Oct3/4	↑	5,19+/-0,9	↓	2,27+/-0,57
Nanog	↑	25+/-13,7	↓	7,21+/-0,17
Mesoderm markers				
Col2A	ND		ND	
Runx2	=	0,07+/-0,03	=	0,052+/-0,08
Neuronal markers				
REST	↓	0,7+/-0,09	↓	0,016+/-0,037
B3-tubulin	↑	1,19+/-0,17	↓	0,68+/-0,094
SCG10	↑	1,17+/-0,01	↓	0,74+/-0,1
Pax6	↓	0,66+/-0,14	ND	
Dlx2	↓	0,44+/-0,17	ND	
GAD 67	ND		Appeared	
DARPP32	ND		Appeared	

**Table 3. Gene expression determined during the differentiation.** Results are expressed in Fold increase and normalized with E/F MIAMI gene expression

## DISCUSSION

In this study we showed that MIAMI cells could be induced towards the GABAergic phenotype *in vitro* and that when transplanted as GABAergic precursors in an *ex vivo* model of HD they survived better when attached to PAMs delivering or not BDNF compared to cells alone. Through this study, we also showed that the *ex vivo* model developed for modelling HD, can be used for evaluating different strategies in the treatment of this disease.

We first determined that LNCs were able to release the siRNA during 6 days explaining the persistent effect of siREST to induce neuronal commitment already observed in normal MSCs. MIAMI cells were first treated with EGF and bFGF as these factors induced their specification towards the neural/neuronal phenotype. Based on Ma et al., 2013 discovery, we decided to further treat MIAMI cells with sonic hedgehog [89] after transfection to obtain LGE-like progenitors. Indeed, the inhibition of REST before the differentiation allowed obtaining functional neurons [2]. In our case, the inhibition of REST during the first step of the differentiation had a favourable effect during all our protocol. Even if siREST is not able to silence REST during fourteen days during the LGE commitment, we clearly observed an increase of cells responding to the treatment during the GABAergic differentiation. Moreover, some evidences suggested that we overpassed the LGE stage with the inhibition of REST. We observed the protein expression of NFM at the end of the commitment, which was not reported previously with SHH treatment. A better characterization should be performed with for example the evaluation of the CTIP2 expression in the LGE-like progenitors obtained. Indeed, in a recent study, CTIP2 was reported as the only transcription factor tested that was able to directly reprogram dermal fibroblasts into DARPP32<sup>+</sup> neurons, suggesting that it a key regulator for GABAergic differentiation [176].

As part of the therapeutic strategy relies on the neuroprotective effect of LM-PAMs-MIAMI-committed cells, we also checked the neuroprotective effect by RT-qPCR *in vitro*. The high expression of VEGFa at the end of the treatment permits to think that these GABAergic-like neurons still retain some characteristics from MIAMI cells, and will be able to have a beneficial effect on

brain slices. It has already been demonstrated that VEGFa possesses angiogenic capacities, and maintained the blood vessels in the in brain slices [84]. Furthermore, during the differentiation VEGFa enhances the gamma-aminobutyric acid (GABA) synaptic activity in embryonic spinal motoneurons [177]. We can easily imagine that VEGFa will induce an improvement of the differentiation of MIAMI cells by stimulating GABAergic synapses. But VEGFa was not the only one secreted by MIAMI derived GABA-like cells, because BDNF and GDNF were also detected in those cells. MSCs secreting BDNF and GDNF have been already described to improve the behavior of rats with HD [145,178,179]. In this way, we can suppose that LM-PAM-LGE-like progenitors will reduce the degeneration. But the major advantage of MIAMI cells is their capacity to express DARPP32, a marker of GABAergic neurons, when grafted attached to LM-PAMs-BDNF. These results are very surprising because when ES cells differentiated into striatal progenitors were grafted in quinolinc acid treated rats, they exhibited a 15% of DARPP32 positive neurons after 13 weeks in situ [37], while a LGE-like progenitors grated reveal a 50% of DARPP32 expression [89]. The advantage of the prolonged release of BDNF by the PAMs should be further investigated. With MIAMI cells we have not quantified yet the pourcentage of DARPP32 expression which could be very interesting in future studies



# **GENERAL DISCUSSION AND CONCLUSION**

Since the discovery of Huntington' disease in 1872, pharmacological treatment or other therapeutic approaches are still insufficient to cure the disease [16]. Progressively, cell therapy became one of the most promising treatments [58]. However, despite many pre-clinical and clinical trials showing functional benefits with this strategy for CNS disorders, it is still not used routinely. In the last twenty years, the implantation of fetal cells for the treatment of HD has demonstrated the possibility of this approach, but shows an important diversity of results in clinical trials due to the heterogeneity in protocols [180,55]. Moreover, the use of ESC or iPS present the risk of over-proliferation in the brain, in addition to the numerous ethical problems previously mentioned [37,90]. Research studies need to focus on resolving the choice of cell type, the number of injected cells and the time of injection. Moreover, the optimization of their survival and differentiation need to be improved before imagining using them in patients. In consequence, in this work, we evaluated an innovative and safe strategy based on regenerative medicine for HD with modified MSCs and appropriate biomimetic scaffold.

In regenerative medicine, an innovative approach is the association of modified cells with therapeutic molecules, morphogens, materials or biomaterials with different physicochemical properties to enhance their biological effects and optimize cell therapy. However, the use of adult stem cells showed numerous limits with a significant death of the implanted cells quickly detected. In addition, relatively low rates of implanted cells differentiate into mature neurons. The capacity of MSCs to undergo functional differentiation into neurons has been questioned over the years and remains controversial [181]. Despite the initial skepticism regarding the capacity of MSCs to differentiate into neurons or glial cells, it was discovered that MSCs express a considerable repertoire of neural genes, which likely contributes to the contested neurogenic predisposition of these cells [182,183]. In addition, bone marrow MSCs express higher neuronal markers [184] compared to MSCs derived from other origins.

In this work, we decided to use a homogenous subpopulation of bone marrow mesenchymal stem cells, MIAMI cells, which have already

demonstrated their capacity to differentiate into dopaminergic neurons with appropriate electrophysiological properties of immature neurons [95,185,88]. Nonetheless, in order to improve the differentiation and their integration in the parenchyma, we chose to knock-down REST. The approach used here is slightly different from strategies generally used for reprogramming MSCs into neurons. Indeed, recent studies have shown that over-expression of transcription factors can serve to promote neuronal differentiation and maintain a neuronal phenotype. Based on the same model to obtain induced Neurons cells (iNs) by forcing the expression of a set of neural lineage transcriptional factors [186], the differentiation of MSCs towards a neuronal lineage has been achieved with Neurogenin1, LMX1 and/or PDX1 (For a review see [187]). Very few studies using the opposite strategy to knock-down and not over-express transcription factors have been performed to improve neuronal commitment[102,175]. In those studies, knock-down of REST was effective with lentivirus and plasmids allowing a stable inhibition of REST.

In this study, we preferred to safely modify the MSCs and focused on transitory inhibition with siREST carried by non-viral vectors to boost the differentiation. We thus designed and characterized two different nanocarriers LNC and SP-AP to efficiently deliver siRNA. Normally, epigenetic manipulation of stem cells is a delicate and complex task. Their remarkable capacity of proliferation makes them poor candidates for non-viral vectors, electroporation and nucleofection, and most stem cells are sensitive to antibiotic selection; hence, attempts to stably establish transfected stem cells are rarely successful [188]. But, in our hands both nanocarriers were perfectly able to delivery the siRNA with a varying degree of toxicity. We also tried to reduce the off-target effect usually described in the literature with cationic lipids for siRNA delivery[189]. These effects are obtained due to silencing of genes other than the intended one and thus compromise the use of a siRNA to study gene function and can even cause cell death. Furthermore, it has been observed that even scrambled sequences, which theoretically do not target any mRNA, can have a moderate to high impact on the cell viability, depending on the cell line and siRNA concentration [190,191]. In order to avoid this, particular care was provided, in this study and in previous ones, to design siRNA nanocarriers with liposomes representing only 20% of the system for the LNCs or without any

cationic lipids in the case of SP-AP nanocarriers [120,192]. Moreover, viability assays were performed with both siREST and siCtle and no difference in terms of toxicity or not differential expression of 2 housekeeping genes was observed which permits us to suppose that the off-target effect is reduced with these nanocarriers.

Both of these systems were recently further optimized and developed. The optimization of the formulation of SP-AP allowed obtaining 70% of siRNA complexation. Furthermore, a new method of detection has been performed to measure the encapsulation efficiency of siRNA-LNC which is now of 75%. Due to their characteristics, the use of other morphogens or therapeutic molecules together with siRNA-nanocarriers can be envisaged. Indeed, during this thesis, a collaborative study allowed determining that the core of LNCs is not used [155] and the loading of different morphogens such as retinoic acid, [193] in order to obtain GABAergic precursors, could be added during the formulation. In this way a synergistic effect on neuronal commitment of stem cells could be obtained. In the same way the siRNA of SP-AP nanocarriers is on the surface letting the matrix core free for addition of growth factors. In other words, combining delivery of a morphogen or a growth factor with a REST knock-down may be a useful strategy for generating functional neurons for therapeutic purposes and easily applied to LNCs or SP-AP. To our knowledge, the co-delivery of siRNA and growth factors with nanoparticles has not been investigated until now in regenerative medicine approaches, but it has already been performed for cancer treatment, demonstrating the possibility to use this approach [194].

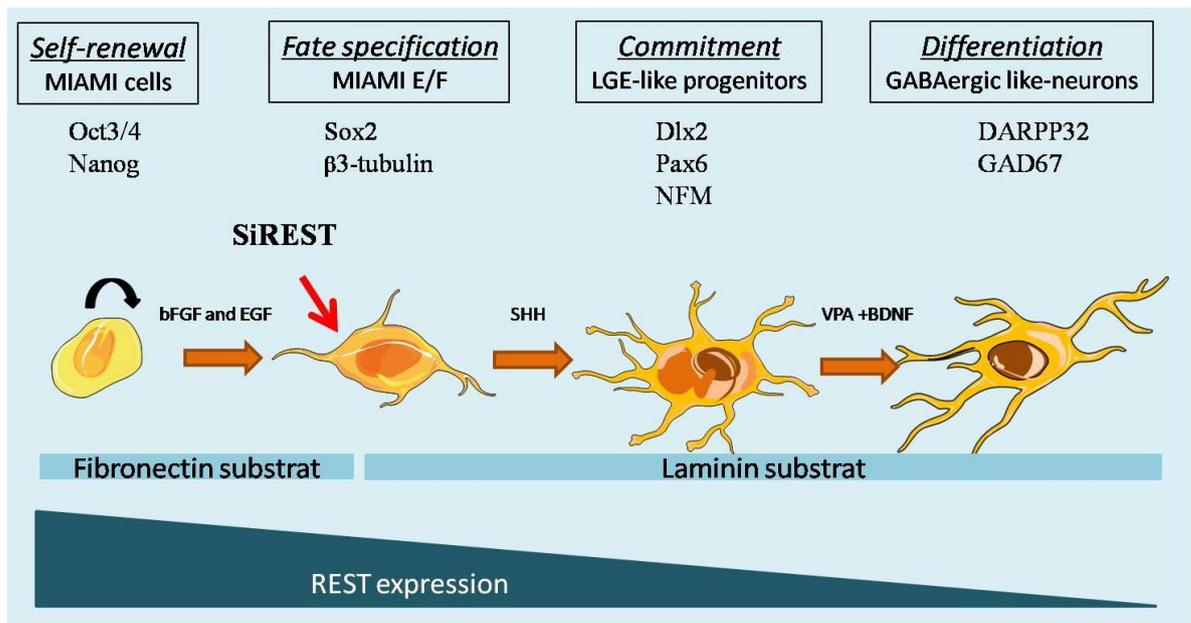
A previous PhD student (Nicolas Daviaud) demonstrated a limited effect of EGF/bFGF pre-treated MIAMI cells in terms of neuroprotection or differentiation in the *ex vivo* HD model. After injecting these MIAMI cells pre-committed towards the GABAergic lineage, he described only 10-15% of stem cells expressing  $\beta$ 3-tubulin, very few committed MIAMI cells expressed DARPP32 and the expression of GAD67 was undetected even when cells were adhered onto LM-PAMs. Based on the literature [37,89,90] we investigated a new 2-step protocol and we characterized the cells obtained four weeks after differentiation *in vitro*. The first step consisted on the neuronal commitment in

order to obtain LGE progenitors, from which MSN derive, and the second one on the final differentiation to obtain these GABAergic-like neurons. Specific media with Sonic Hedgehog was used during fourteen days after siREST transfection. SHH is a pleiotropic factor for the development of CNS driving proliferation, specification, and axonal targeting in multiple sites within the forebrain, hindbrain, and spinal cord. Studies in embryonic CNS have shown how gradients of this morphogen are translated by neuroepithelial precursors to determine the types of neurons and glial cells they produced [195]. Using these conditions LGE progenitors were obtained [89] (**Figure 12**). Nonetheless, in our work, we concluded that we over-passed this stage with the high expression of NFM and  $\beta$ 3-tubulin as well as the decrease of DLX2 at the end of the commitment. In order to better understand those results the mechanism and signaling forced by the inhibition of REST must be elucidated.

REST expression is very high in ESCs compared to most other cell types[152], and is thought to be involved in their self-renewal. REST is regulated directly by the pluripotent factors Oct4/Sox2 and Nanog, maintaining a high expression of REST, but its function in the self-renewal transduction network is unclear [196–198]. In this work, the inhibition of REST without any other differentiated media was enough to induce neuronal commitment of MSCs, which is not surprising due to the functionality of REST [151,151]. But, at the end of this period we still observed a slight diminution of REST. This inhibition is not only explained by the prolonged release of REST with LNCs, because we did not observe any siRNA after 6 days (data not shown). We thus assume that siREST helped and accelerated the commitment as described in mouse previously [198,199] and during neurogenesis *in vivo*[200] However, various other explanations can be advanced. One explanation relates to the levels of pluripotent factors such as Nanog. Indeed, the level of Oct4 and Nanog is lower in EGF/bFGF pre-treated MIAMI cells compared to non pre-treated MIAMI cells[185] and they should continue to decrease during the differentiation. In this way, they are unable to maintain or re-activate high levels of REST within the cell. The microenvironnement is also very important for REST regulation. In a recent publication, Shing et al., investigated the role of laminin on pluripotency and demonstrated the reduction of self-renewal of ESCs on laminin coated surfaces [201]. In this way, we chose to transfect MIAMI cells after

laminin adherence because laminin coated surfaces also play a modest role in the loss of pluripotency [201]. Moreover, *in vivo* reports describe the role of laminin in axonal regeneration of the CNS [202]. In our study, proliferation seems to diminish after transfection with siREST and some neurites appeared during the commitment step with SHH but in order to confirm those data the possible interaction between REST and kinase or cyclin proteins involved in the cell cycle needs to be explored. Finally, REST activity represses the expression of GluR2 in neurons destined to die, indicating that REST might play an important role in insult-induced death of neurons[203] [204].

The final differentiation step allowed obtaining GABAergic-like neurons *in vitro*. The best condition was a 4 weeks treatment with the supplementation of Valproic acid and BDNF at the same time (**Figure 12**). Those results will be confirmed with repeated experiments, but they established the choice of BDNF as the growth factor to be delivered by the PAMs for the grafting of committed MIAMI cells and PAMs. Accordingly, laminine was confirmed as the best molecule for functionalizing the surface of the PAMs. In this way we chose to graft the EGF/bFGF MIAMI cells after commitment towards GABAergic precursors with siREST LNCs and SHH with or without laminine PAMs delivering or not BDNF. We already successfully showed the regenerative effect of EGF/bFGF MIAMI cells and PAMs releasing NT-3 in a PD model [84,88,174]. In addition, the transduction pathway allowing NT-3 to induce differentiation into a neuronal cell type was studied *in vitro* and it was demonstrated that the fixation of NT-3 to its receptor stimulated the kinase pathway MEK / ERK. Moreover, the action of Rac1b on this pathway to block proliferation and stimulate the development of neurites was also elucidated [205]. So, strong of those results, we could intend a similar strategy for HD.



**Figure 12.** The different steps observed during *in vitro* differentiation

The preliminary evaluation of this combined regenerative medicine strategy has been performed with innovative organotypic brain slices modeling the GABAergic degeneration observed in HD. Indeed, taking into account the numerous advantages of organotypic brain slices, we developed a simple model of HD, where GABAergic MSNs degeneration is due to mechanical transection of the axonal pathway between the striatum and substantia nigra. We wanted to develop a simple, inexpensive and mono-parametric model of HD. Based on the same principle that we have already developed to model Parkinson's disease [206], we demonstrated that the axis of sectioning is very important as only coronal sections allowed modeling the progressive degeneration of MSNs. The slices were viable at least until day 19 as determined by Neu N expression as well as the MSN degeneration determined by the decrease of DARPP32 and GAD67 expression. The mechanism of degeneration is still poorly understood in this model, and we hypothesize that the regulation of the physiological state of these striatal neurons via dopamine is impaired. Nonetheless, the determination of the dopamine-regulated ion channels could be performed by electrophysiological studies. In addition, a possible reduction in the striatal-specific dopamine targets, PKA, DARPP-32, ARPP-16, and ARPP-21 could be performed, with RT-qPCR or immunofluorescence.

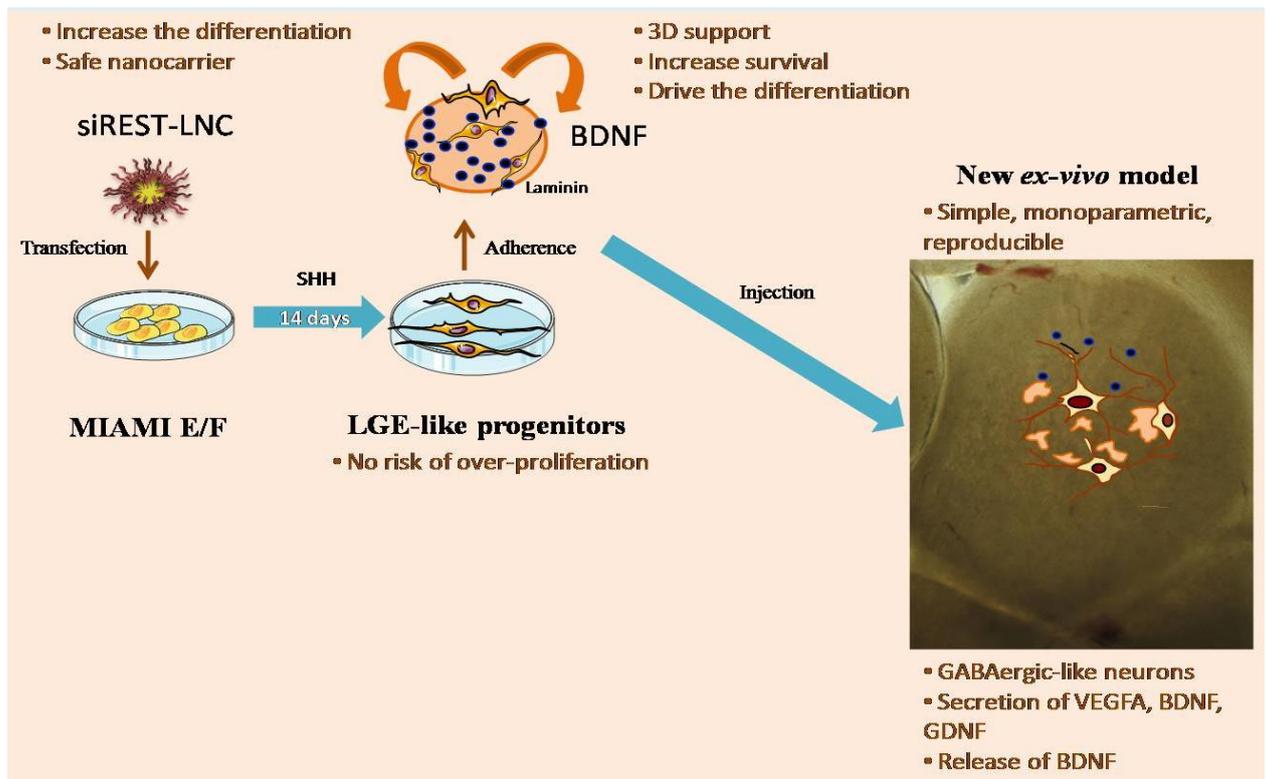
This *ex vivo* model will allow screening the best strategy for HD and further comprehending the mechanisms involved in the regeneration of the GABAergic neurons as performed with an *ex vivo* model of PD by our group [206]. For example, we can interrogate the amount of cells to be injected and if they should be injected as a bolus or repeated injections. Furthermore, the evaluation of the behavior of stem cells after grafting must be performed at long-term. Therefore, we are going to follow NeuN and GAD67 over-time until 30 and 60 days to evaluate the long-term viability of the slices and follow the degeneration of the GABAergic neurons. A recent study allowed observing few axonal projections (0.017%) after 9 weeks injection of differentiated ESC and more axonal projections after in utero implantation, suggesting integration of the donor cells into the neuronal network of the host brain [207]. But the poor quantity of axonal projection demonstrated the difficulty to hope replace GABAergic neurons. Although transplanted cells integrated and were able to reduce the motor asymmetry in the QA-lesioned model, long-term studies are needed to assess circuit reconstruction and behavioral recovery. With our model, we can obtain cortico-striatal brain slices or the whole coronal section. In this way, we can screen the different regenerative medicine strategies and select the best approach showing benefits in terms of neuroprotection, not only in the striatum but also in the cortex and the globus pallidus, which are involved in HD.

In adult PD rat brain, we have first shown that the 60  $\mu\text{m}$  size and PLGA-PAMS could be stereotactically implanted in precise areas of the brain without causing damage to the surrounding tissue. Nonetheless in organotypic slices, we thought that 30  $\mu\text{m}$  could be better. So, in this study, the triblock copolymer poloxamer P188 ((poly (ethylene oxide) (PEO)–poly(propylene oxide) (PPO)–PEO) linked to PLGA to form this PLGA-P188-PLGA co-polymer was used to formulate PAMs with 30  $\mu\text{m}$  size. Indeed, PLGA based implantable devices are FDA approved [208] but the efficient and sustained release of proteins from PLGA-PAMs remains a challenge, mainly due to protein instability (adsorption, aggregation, and denaturation) during the formulation process or the release period. Therefore this polymer was used to formulate the PAMs in this study. Interestingly, PLGA-P188-PLGA 60  $\mu\text{m}$  microsphere releasing TGF $\beta$ 3, developed for cartilage tissue engineering, resulted in a prolonged and almost

complete TGF $\beta$ 3 release [209]. They offered the advantage to limit protein adsorption but also to stabilize the pH inside the microsphere thus preventing protein unfolding and denaturation. Moreover, an increase in MSC number was observed after 7 days in culture on fibronectin-covered PLGA-P188-PLGA PAMs compared to fibronectin-covered PLGA PAMs.

In our study the 3D support offered by the scaffold allowed cell retention within the striatum and, probably cell survival as seen by the increased number of human cells with the PAMs compared to cells implanted alone. However, BDNF-PAMs also seemed to drive cell differentiation of pre-committed MIAMI cells, demonstrated by the increase of DARPP32 markers compared to Blank-PAMs. These results are in accordance with previous reports showing that BDNF increases DARPP32 expression in neurons of the striatum [210,211]. Moreover, it appears to induce significant neuroprotection of NMS neurons in *in vivo* models of Huntington's disease [212,213]. A collaborative work allowed determining the release of BDNF from PAMs, which corresponded perfectly to the amount (15 ng/day) needed by the cells for their differentiation[37,89,90] Nonetheless, we don't know if the protective effect on MIAMI cells is due to the BDNF released from PAMs or BDNF secreted by MIAMI cells. The neuroprotective effect of MIAMI on the GABAergic neurons is also another important part of this project. In the present study, in preliminary results, we observed *in vitro* that MIAMI cells expressed BDNF and GDNF as well as a very high level of hVEGFA. BDNF and GDNF have both been reported as neuroprotective factors whose effects were, in part, mediated by autophagy regulation[214] and oxidative stress diminution[215,216], respectively. The neuroprotective effect of BDNF and GDNF has been evaluated in pre-clinical studies in HD [217–219]. In this way, pre-committed MIAMI cells are good candidates for neuroprotection. hVEGFA is playing a dual role, indeed, hVEGFA can increase grafted cells survival by creating new blood vessels around the graft enhancing oxygen and nutrients supply and a neuroprotective effect. Indeed, in a more recent study, the delivery of hVEGFA via injectable hydrogels, to rats receiving striatal injection of quinolinic acid to mimic neuronal loss and behavioral deficits characteristic of HD, was shown to be neuroprotective at 3 weeks [220]. As hVEGFA is well-known to have pro-angiogenic effect, a quantification of blood vessels in the brain slices should be

performed. Complete analyses of the secretome of MIAMI cells are going on in order to better characterize proteins secreted by LGE-like progenitors.



**Figure 13.** Schematic strategy finally used during this thesis and some advantages.

However, a final assessment of this strategy includes the behavioral evaluation in an appropriate *in vivo* model. In this work, intracranial injection of MIAMI cells is envisaged and we think it is the best option as the cells and PAMs need to be close to their site of action to avoid undesired side-effects. We can also consider delivery of siREST by implantation of the si-REST nanocarriers together with the cell graft. Indeed, REST expression is increased in HD [221,222] and its association with HTT in the cytoplasm is disrupted in HD due to the mutated HTT. This leads to increased nuclear REST and concomitant repression of several neuronal-specific genes, including BDNF [67]. The finding described indicates that inhibition of REST expression may offer a new therapeutic avenue for treatment of HD. Nowadays, innovative strategies including the use of RNAi to reduce REST expression [223] or decoy oligonucleotides or synthetic peptide nucleic acid (PNA) oligomers to sequester REST away from its genomic sites [224,225] are underway. All of these approaches lead to reduce interactions of REST with its target genes; however,

translation of this into a useful therapeutic strategy requires safe and efficient delivery of these tools to the brain of HD patients. This is no easy task and LNCs or SP-AP could be used for that, but this strategy needs to be carefully analyzed. More particularly, a full characterization of this combined strategy will be evaluated during a post-doctoral contract.



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# ANNEXES

# **PUBLICATION**

## Research Article

## Efficient *in vitro* gene therapy with PEG siRNA lipid nanocapsules for passive targeting strategy in melanoma

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Small interfering RNA (siRNA)-mediated gene therapy is a promising strategy to temporarily inhibit the expression of proteins implicated in carcinogenesis or chemotherapy resistance. Although intra-tumoral administration can be envisaged, studies currently focus on formulating nanomedicines for intravenous injection to target tumor sites as well as metastases. The development of synthetic nanoparticles and liposomes has advanced greatly during the last decade. The objective of this work consists in formulating and optimizing the encapsulation of siRNA into lipid nanocapsules (LNCs) for efficient gene therapy to target melanoma cells. siRNA LNCs were prepared from DOTAP/DOPE lipoplexes, and the siRNA amount and lipid/siRNA charge ratio were assayed to improve the stability and the encapsulation yield. Cryo-TEM imaging of the siRNA lipoplexes and LNC morphology revealed specific organization of the siRNA DOTAP/DOPE lipoplexes as well as specific lipid microstructures that can be eliminated by purification. No cytotoxicity of the siRNA LNCs against the melanoma SK-Mel28 cell line was observed at concentrations of up to 500 ng/mL siRNA. *In vitro* siRNA transfection experiments, compared to Oligofectamine™, demonstrated interesting targeted gene silencing effects. Finally, complement activation assays confirmed the feasibility of the PEGylation of siRNA LNCs as part of a passive targeting strategy for future *in vivo* melanoma- and metastasis-targeting experiments.

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### 1 Introduction

Gene therapy is a rapidly emerging field. Initial gene therapy strategies sought to increase the expression of deficient genes in monogenic diseases by the efficient

transfection of DNA plasmids. A more recent gene therapy strategy aims to transiently inhibit the expression of a target gene in chronic diseases such as cancer. In this strategy, the use of a small interfering RNA (siRNA), in place of a DNA plasmid, produces a partial or total extinction of both messenger RNA (mRNA) and protein levels of the targeted gene [1]. Proof of concept of siRNA action has been provided by commercial transfection agents that can suppress the expression of proteins *in vitro* in tumor cell cultures, resulting in decreased tumor cell proliferation [2, 3]. However, the application of this strategy is limited by the ability to systemically inject these nucleic acids. In light of this, our objectives were to obtain: (i) high encapsulation and stability of siRNA in the nanocarrier; (ii) efficient protection from the innate immune system present in the blood; and (iii) improved uptake into targeted cells with efficient transfection.

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**Abbreviations:** CR, Charge ratio; **Cryo-TEM**, Cryogenic transmission electron microscopy; **DLS**, Dynamic light scattering; **DOPE**, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; **DOTAP**, 1,2-dioleoyl-3-trimethylammonium-propane; **EDOPC**, dioleoyl *O*-ethyl-dioleoylphosphatidylcholine; **EE**, Encapsulation efficiency; **EBB**, Ethidium bromide; **LNCs**, Lipid nanocapsules; **MTS**, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium; **NHS**, Normal human serum; **PEG**, Polyethylene glycol; **PIT**, Phase inversion temperature; **siRNA**, Small interfering RNA

Initial studies involved viruses such as cytomegalovirus (CMV) or associated adenoviruses (AAV). However, high costs, risks of genome rearrangement, possible crossing with natural viruses, and inflammatory and immunogenic reactions have limited the use of these viral forms and encouraged the development of non-viral carriers, such as synthetic nanoparticles. Over the last few years some nanomedicines have been formulated to efficiently encapsulate siRNA and deliver it into the blood stream without degradation [4]. Nanoparticles and liposomes are the most studied of these siRNA delivery systems. Nanoparticles with an oily core and a monolayer shell are usually based on polymers such as polyethylene glycol (PEG) and can protect nucleic acids from degradation by nucleases and cells belonging to the innate immune system [5–7]. Liposomes composed of bilayers of cationic charged lipid components, such as cholesterol, DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), or DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), enable: (i) the fixation of siRNA by electrostatic forces; (ii) the interaction of siRNA with cell membranes improving the cellular uptake; and (iii) the endosomal escape of the siRNA [8–10]. Polymers and lipids have frequently been employed in tandem in the formulation of new siRNA delivery systems to combine the advantages of both components, such as in the siRNA lipid nanocapsules (siRNA LNCs) presented in this study.

LNCs, which consist of a lipid liquid core of triglycerides and a rigid shell of lecithin and PEG, were developed in our laboratory [11]. The simple formulation process is based on the phase inversion of an emulsion. At first LNCs were used to load DNA [12–15]. Recently these LNCs have been modified to encapsulate siRNA complexed with DOTAP/DOPE lipids resulting in efficient encapsulation of up to 60% siRNA [16]. Transfection of U87MG glioma cells with these siRNA LNCs resulted in inhibition of EGFR protein expression by 63% [17]. In the present study, siRNA LNCs were characterized by specific microscopic methods to provide insights into siRNA organization and LNC structure. Moreover, the process was optimized to improve the siRNA payload and the stability of siRNA LNCs over time. The cytotoxicity of the siRNA LNCs towards the SK-Mel28 human melanoma cell line was then assessed. The final aim was to produce a partial or total extinction of mRNA and protein levels of the targeted gene using siRNA LNCs. Therefore, the *in vitro* experiment examining the ability of LNCs carrying siRNA to transfect cells and suppress protein levels of the targeted gene was performed. Finally, with the aim of designing stealth nanocarriers for *in vivo* systemic administration, the addition of long PEG chains at the LNC surface was evaluated by complement activation assays.

## 2 Materials and methods

### 2.1 Formulation of siRNA LNCs

#### 2.1.1 Liposomes and lipoplexes

For liposome preparation, the cationic lipid DOTAP (Avanti® Polar Lipids Inc., Alabaster, AL, USA) was dissolved in chloroform and combined at a 1:1 molar ratio with the neutral lipid DOPE (Avanti® Polar Lipids Inc., Alabaster, AL, USA) to obtain a final concentration of 30 mM cationic lipid charge based on the number of lipid charges per molecule, i.e., 1 for DOTAP. After evaporation of chloroform under vacuum, deionized water was added to rehydrate the lipid film overnight at 4°C. The lipid film was then sonicated for 30 min.

Lipoplexes were formulated as a simple equivolume mixture of siRNA and liposomes. The complexes were characterized by their charge ratio (CR) [17], i.e., the ratio between the positive charges of the lipids and negative charges of the nucleic acids (+/- ratio), which ranged from 5 to 15.

#### 2.1.2 siRNA LNCs

LNCs were formulated as described previously [11] by combining 20.5% w/w Labrafac WL 1349 (caprylic-capric acid triglycerides, Gatefossé S.A. Saint-Priest, France), 1.5% w/w Lipoid S75-3 (Lipoid GmbH, Ludwigshafen, Germany), 16.9% w/w Kolliphor® HS 15 (BASF, Ludwigshafen, Germany), 1.8% w/w NaCl (Prolabo, Fontenay-sous-Bois, France), and 59.8% w/w water (obtained from a Milli-Q system, Millipore, Paris, France) with magnetic stirring. The temperature was cycled three times between 60 and 95°C to produce phase inversions of the emulsion. Subsequent rapid cooling and dilution with ice-cooled water after the last phase inversion temperature (PIT) led to the formation of blank LNCs.

To obtain siRNA LNCs, the aqueous solution was replaced during the last temperature cycle by aqueous solution containing lipoplexes, i.e.,  $\alpha 1$  subunit siRNA (sense sequence: 5'-GGGCAGUGUUUCAGGCCUUATT-3'; antisense sequence: 5'-UUAGCCUGAAACACUGCCCTT-3'; Eurogentec, Seraing, Belgium), and scramble or control siRNA (sense sequence: 5'-UCUACGAGGCACGAGACUUTT-3'; antisense sequence: 5'-AACUCUCGU GCCUCGUAGATT-3') complexed with cationic liposomes in a defined CR, as described above.

### 2.2 Surface modification by post-insertion

The polymer used for post-insertion was 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000) (Mean Molecular Weight (MMw) = 2805 g/mol) (Avanti Polar Lipids, Inc., Alabaster, USA). This polymer was added to LNCs to obtain a final concentration of 10 mM DSPE-mPEG2000. Pre-formed siRNA LNCs and DSPE-mPEG2000 were

co-incubated for 4 h at 37°C. The mixture was vortexed every 15 min and then quenched in an ice bath for 1 min.

### 2.3 Filtration and purification

After formulation and/or the DSPE-PEG2000 post-insertion, the LNCs were filtered with a 0.2 µm filter (Acrodisc PALL GHP, VWR, Radnor, USA) or purified using PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France). Purifications were performed in water or with low concentrations of sodium hydroxide (0.15 M) to disrupt the interaction between the lipids and siRNA. After this purification step, the salt concentration of the suspension was adjusted to a physiological NaCl concentration (150 mM).

### 2.4 Characterization of siRNA LNCs

#### 2.4.1 Size and zeta potential

Dynamic light scattering (DLS), using a Malvern Zetasizer<sup>®</sup> apparatus (Nano Series ZS, Malvern Instruments S.A., Worcestershire, UK), was used to measure the size and zeta potential of the LNCs in triplicate at 25°C after dilution to a ratio of 1:200 in deionized water [18]. These parameters were measured daily over the course of one month.

#### 2.4.2 Encapsulation efficiency (EE)

##### 2.4.2.1 Qualitative ethidium bromide (EtB) electrophoresis detection

The EE and integrity of the siRNA molecules after nanocapsule formulation and post-insertion were evaluated by EtB agarose gel electrophoresis. Samples of LNC suspension equivalent to 2.5 µg of siRNA were tested with or without Triton<sup>®</sup> X100 (Sigma, Saint-Quentin Fallavier, France), mixed with 5 µl gel-loading solution (Sigma, Saint-Quentin Fallavier, France) and loaded into each well of a 1% agarose gel containing 0.05 µg/mL of EtB (Sigma, Saint-Quentin Fallavier, France) [12]. Free siRNA (2.5 µg) in solution was used as a control. The samples were allowed to migrate for 20 min at 125 V in a 1× Tris-EDTA buffer (Sigma, Saint-Quentin Fallavier, France).

##### 2.4.2.2 Quantitative UV detection

A spectrophotometric method based on recent work by David et al. was used to evaluate the EE [16]. Briefly, siRNA LNCs were mixed with chloroform and water to separate hydrophilic and lipophilic components, respectively. Sodium hydroxide was then added to destabilize the lipoplexes, and finally, absolute ethanol was added to destroy the LNCs [16]. After two centrifugation steps, four fractions were obtained: (i) free siRNA; (ii) free lipoplexes (i.e., siRNA associated with liposomes); (iii) siRNA encapsulated into LNCs; and (iv) lipoplexes encapsulated into

LNCs. To determine the concentration of siRNA, the optical density of each sample was determined in triplicate at 260 nm (UV-2600, Shimadzu, Noisiel, France). The EE was measured every week for one month using these two methods.

### 2.5 Cryogenic transmission electron microscopy (cryo-TEM) [19]

Specimens for cryo-TEM imaging were prepared using a cryo-plunge cryo-fixation device (Gatan, Pleasanton USA) in which a drop of the aqueous suspension was deposited on a carbon-coated grid (Ted Pella Inc., Redding, USA). The TEM grid was then prepared by blotting the drop containing the specimen so that a thin liquid layer remained across the holes of the carbon support film. The liquid film was vitrified by rapidly plunging the grid into liquid ethane cooled by liquid nitrogen. The vitrified specimens were mounted in a Gatan 910 specimen holder (Gatan, Pleasanton, USA), which was inserted into the microscope using a CT-3500-cryotransfer system (Gatan, Pleasanton, USA), and cooled with liquid nitrogen. TEM images were obtained from specimens preserved in vitreous ice and suspended across a hole in the supporting carbon substrate [20, 21]. The samples were observed under low-dose conditions ( $<10\text{ e}^{-}/\text{Å}^2$ ), at  $-178^{\circ}\text{C}$ , using a JEM 1230 “Cryo” microscope (Jeol, Akishima, Japan) operated at 80 kV and equipped with a LaB<sub>6</sub> filament. All micrographs were recorded on a Gatan 1.35K × 1.04K × 12 bit ES500W CCD camera.

### 2.6 Drop tensiometry

Oil-water interface (Labrafac<sup>®</sup>-water) behavior was measured using a drop tensiometer (Tracker, ITConcept, Longessaigne, France) and a water drop containing different quantities of liposome, lipoplex, or siRNA in bulk Labrafac<sup>®</sup> [22]. The quantities of liposome and lipoplex were calculated based on the masses used in the formulation of the LNCs. The concentrations tested ranged from 2 to 12 mg of lipid per gram of Labrafac<sup>®</sup>, and the observed lipid concentration in siRNA LNCs was 11.36 mg/g. A 5 µL ( $\sim 12.5\text{ mm}^2$ ) rising drop of water, with or without liposomes or lipoplexes, was formed using an Exmire microsyringe and a gauge (Prolabo, Paris, France) in a glass vial filled with an oily phase (Labrafac<sup>®</sup>). The axially symmetric shape (Laplacian profile) of the drop was analyzed using a camera connected to a computer. From the analysis of the digital image and integration of the drop profile according to the Laplace equation, the interfacial tension and surface area could be simultaneously calculated and recorded in real time. The volume was controlled by a motor operating the micro-syringe. To enable adsorption of liposomes and lipoplexes onto the interface, the drop was equilibrated for 12 h (at 25°C), with the surface area kept constant with the help of the syringe motor.

At the end of the equilibration time, the equilibrium surface tension was recorded.

## 2.7 Human SK-Mel28 melanoma cell culture

Human SK-Mel28 melanoma cell lines were obtained from ATCC (LGC Promochem, Molsheim, France). The cell lines were grown in RPMI medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium), 1% antibiotics (10 units of penicillin, 10 mg of streptomycin, and 25 µg of amphotericin B/mL; Sigma-Aldrich, St. Louis, USA), and 1% non-essential amino acids (Lonza). Cell lines were cultured according to the ATCC protocol and maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

## 2.8 Viability assay

Cells were seeded onto 24-well plates at a density of  $5 \times 10^4$  cells/well and precultured overnight. Prior to incubation, the medium was replaced with fresh medium without serum. The cells were treated with blank or siRNA LNCs at siRNA concentrations of 50 to 500 mg/mL for 4 h. After removing the LNCs, the cells were cultured for two days before determining the IC<sub>50</sub>. Cytotoxicity assays were performed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) (Promega, Madison, USA). Each well contained 100 µL of MTS, and the plates were incubated for 2.5 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The OD was measured at 492 nm with a Multiskan Ascent (Labsystems, Fisher Scientific, Wilmington, USA).

## 2.9 Transfection experiments

SK-Mel28 cells were seeded onto 6-well plates at the density of  $2 \times 10^5$  cells/well or in flask (25 cm<sup>2</sup>) with  $5 \times 10^6$  cells/flask and precultured overnight. Before transfection, the medium was replaced with fresh medium containing no serum. Different treatments (control, scramble siRNA LNCs, and alpha 1 siRNA LNCs) were incubated with cells and removed 8 h post-transfection. The positive control was realised with Oligofectamine™ according to the manufacturers protocol using scramble and alpha 1 siRNA. Transfection was realised on three consecutive days.

## 2.10 Western blot

After three transfections, total protein was extracted from melanoma cells by scraping in the presence of a cell lysis buffer (10 mmol/L Tris-Base, 1 mmol/L Na<sub>3</sub>O<sub>4</sub>, and 1% SDS, pH 7.4) and storing at -20°C. Twenty micrograms of protein were resolved on a 10% (v/v) SDS-PAGE gel and then transferred to a nitrocellulose membrane (0.45 µm pore size) (Amersham GE Healthcare). Mouse anti-human

Na/KATPase α1 (clone C464.6; Millipore) and mouse anti-human actin (Clone C4; Millipore), for use as loading controls, were diluted to the respective ratios of 1:1000 and 1:5000 according to the manufacturer's instructions and incubated over night at 4°C. A second mouse antibody (Dako) was used at a dilution of 1:2000. Detection (using LAS4000, GE HealthCare) was performed using enhanced chemiluminescence (ECL; Fisher Scientific, Pierce).

## 2.11 RT-qPCR

After three transfections, total RNA was extracted and purified using RNeasy Microkit (Qiagen, Courtaboeuf, France), and treated with DNase (10 U DNase I/µg total RNA). RNA concentrations were determined using a ND-2000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA) and the values obtained used for normalization of the RNA template in the reverse transcription reactions. First-strand cDNA synthesis was performed with a SuperScript™ II Reverse Transcriptase (Invitrogen), in combination with random hexamers, according to the manufacturer's instructions. Following first-strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 40 µL RNase-free water (Gibco). 3 ng of cDNA was mixed with Maxima™ SYBR Green qPCR master mix (Fermentas) and primer mix (0.3 µM) in a final volume of 10 µL. Amplification was carried out on a LightCycler 480 (Roche) with a first denaturation step at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 30 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. A mean cycle threshold value (Cq) was obtained from 2 measurements for each cDNA. Specific gene expression was calculated using the 2<sup>-ΔΔCT</sup> method using GAPDH as calibrator.

## 2.12 CH50 assay

Complement consumption was assessed in normal human serum (NHS) (provided by the Etablissement Français du Sang, Pays de la Loire – Angers, France) by measuring the residual hemolytic capacity of the complement system after contact with LNCs. The final dilution of NHS in the mixture was 1:4 (V/V), corresponding to 100 µL of NHS in 400 µL of reactive media. The amount of serum required to lyse 50% of a fixed number of sensitized sheep erythrocytes using rabbit anti-sheep erythrocyte antibodies (CH50) was determined according to a procedure described previously [23]. Complement activation was first expressed as a function of the surface area of the LNCs and then used to compare different formulations. Nanoparticle surface areas were estimated using the equation:  $S = 3m/\rho r$ , where S is the surface area [cm<sup>2</sup>], m is the weight [g] in 1 mL of suspension, r is the average radius [cm] as determined by DLS, and ρ is the volumet-

ric mass [ $\text{g}/\text{cm}^3$ ] of the nanoparticles estimated at  $1 \text{ g}/\text{cm}^3$ .

### 3 Results and discussion

#### 3.1 siRNA LNC characterization

##### 3.1.1 CR and siRNA amount influence the stability of siRNA LNCs

LNCs were prepared with three different amounts of lipids/siRNA CR (Fig. 1). At day 0, all samples had an LNC size of approximately 110 nm with a polydispersity index (PDI) of less than 0.3 (Fig. 1A). Over time the size of the LNCs decreased, depending on the amount of siRNA, to a minimum size of 60 nm. The size stabilized in 7 to 14 days for high and low amounts of siRNA, respectively. A direct correlation between size and zeta potential was observed (Fig. 1A). While size decreased, zeta potential increased up to +30 mV, confirming siRNA LNC reorganization. Despite perturbation of size and zeta level, the yield was constant for 1 month, indicating a strong interaction between the siRNA and LNCs (Fig. 1E). Interestingly, the siRNA-encapsulated yield differed depending on the amount siRNA introduced into the formulation (Fig. 1F). Saturation of siRNA incorporation during the encapsulation process was not observed below  $750 \mu\text{g}$  siRNA, leading to an estimated maximal EE of 53%, which corresponds to  $130 \mu\text{g}$  siRNA per mL of LNC suspension. Many formulations reported in the literature that have been based on electrostatic interactions, such as with lipoplexes, have a yield greater than 90% complexation after electrophoresis or centrifugation [24, 25]. However, our siRNA LNC synthesis more closely resembles calcium phosphate (CaP) nucleic acid nanoparticle synthesis developed by Zhang et al. Similar to our findings, this process includes a nanoencapsulation step for lipoplexes and leads to a yield of 55% (radioactive dosage with tritium) [26].

A positive CR greater than 3 has previously been used for complexation of siRNA and lipids [27]. In initial studies of siRNA LNC formulation, superior encapsulation was observed for DOTAP/DOPE lipoplexes with a CR of 5 compared to those with a CR of 2.5 [16, 17]. In the present study, no significant improvement of the EE or stability was observed for a CR of 10 (Fig. 1B and F). Unexpectedly, the highest CR tested (15) led to small nanocapsules of approximately 50 nm and no encapsulation of siRNA (Fig. 1B and F). At a high CR, the substantial excess of positive charges seems to prevent efficient siRNA encapsulation. Consequently, the large size (100 nm) obtained at day 0 for lower CRs might indicate siRNA incorporation into LNCs. The quantities of siRNA and liposomes used to formulate the LNCs appears to be a key factor in improving the stability and EE of siRNA LNCs. Based on these results, a medium siRNA content of  $375 \mu\text{g}$  and a low CR

(5) were selected as the conditions for subsequent experiments.

##### 3.1.2 Evaluation of siRNA stability in response to manufacturing and storage conditions

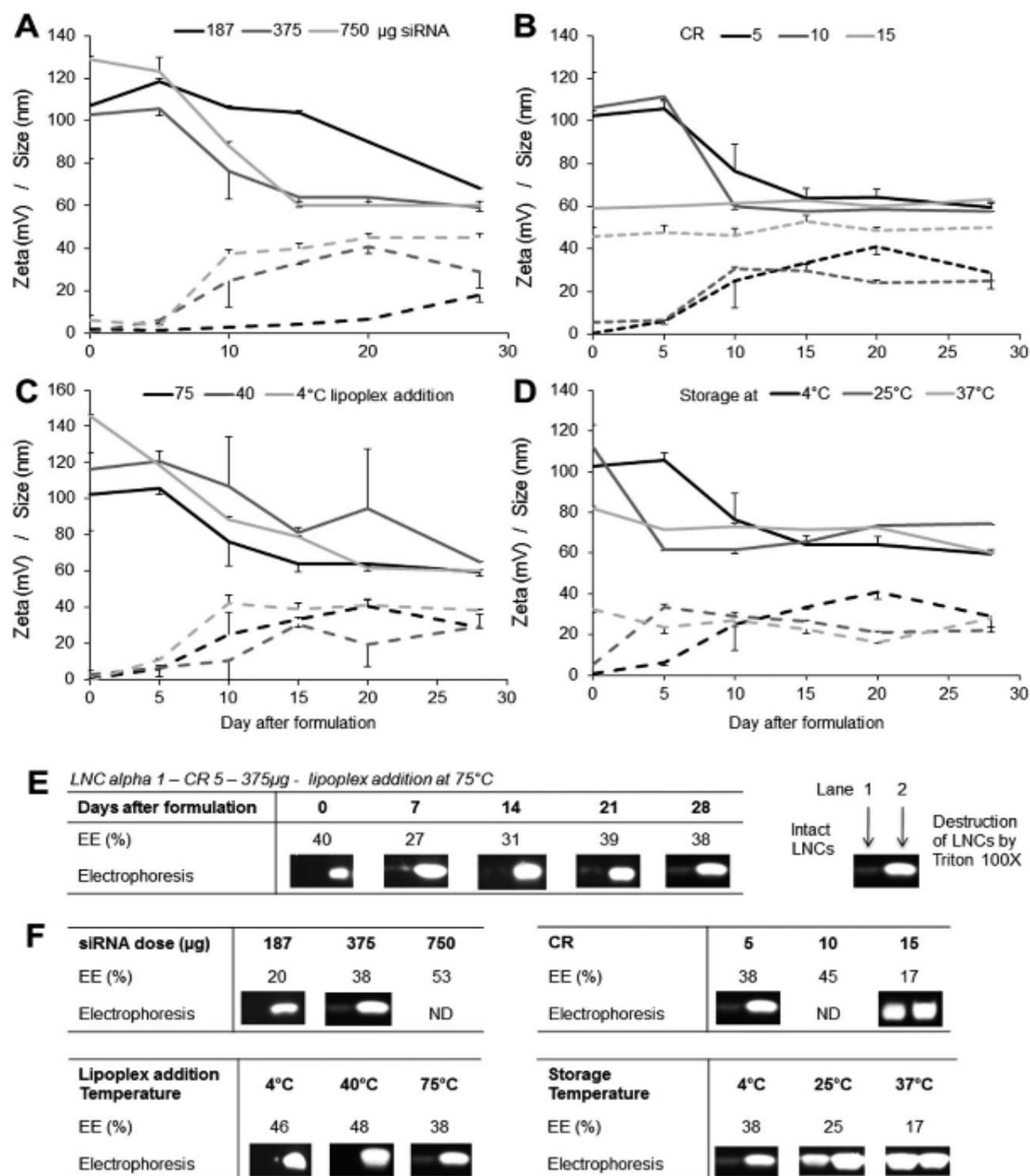
Storage temperature is a key parameter in ensuring the stability of nanomedicines over time with scale-up and industrialization of the process. Temperatures of 25 and  $37^\circ\text{C}$  were tested to predict the behavior of siRNA LNCs at room and physiological conditions, respectively (Fig. 1D). Our results demonstrated the necessity of  $4^\circ\text{C}$  storage compared to room or physiological temperature in order for the siRNA to be retained in the LNCs (Fig. 1F). Room and physiological temperatures induced a rapid decrease in LNC size, and electrophoresis studies revealed that siRNA delivery began 8 h after incubation and was complete at 24 h at  $37^\circ\text{C}$  (data not shown). In this case, the reduction in size was associated with siRNA delivery and demonstrated the reversibility of the electrostatic interactions between the siRNA and LNCs. As a result of these findings the siRNA LNCs were stored at  $4^\circ\text{C}$ .

To avoid the possible denaturation of siRNA during the process, lipoplexes were added during the third temperature cycle at the PIT, which corresponded to  $75^\circ\text{C}$ , which is the dilution temperature typically used in the LNC formulation process. Lipoplexes were also introduced at lower temperatures: at  $40^\circ\text{C}$ , during the phase inversion at  $75^\circ\text{C}$ ; and at  $4^\circ\text{C}$  with stirring after the completion of blank LNC formation. No major differences in physico-chemical characteristics or EE levels were observed at the varying temperatures (Fig. 1C and F), indicating that the encapsulation of siRNA by LNCs is not dependent on the dilution temperature. This result suggests that lipoplexes can interact with LNCs during and/or after complete LNC formation, indicating that lipoplexes are most likely located within the shell, at the interface between the LNC core and water. Similarly, Pitaksuteepong et al. also reported an interfacial localization of hydrophilic compounds when introduced into a water-in-oil emulsion [28].

#### 3.2 siRNA LNC structure

##### 3.2.1 Tensiometry study: Evidence of interfacial localization of siRNA

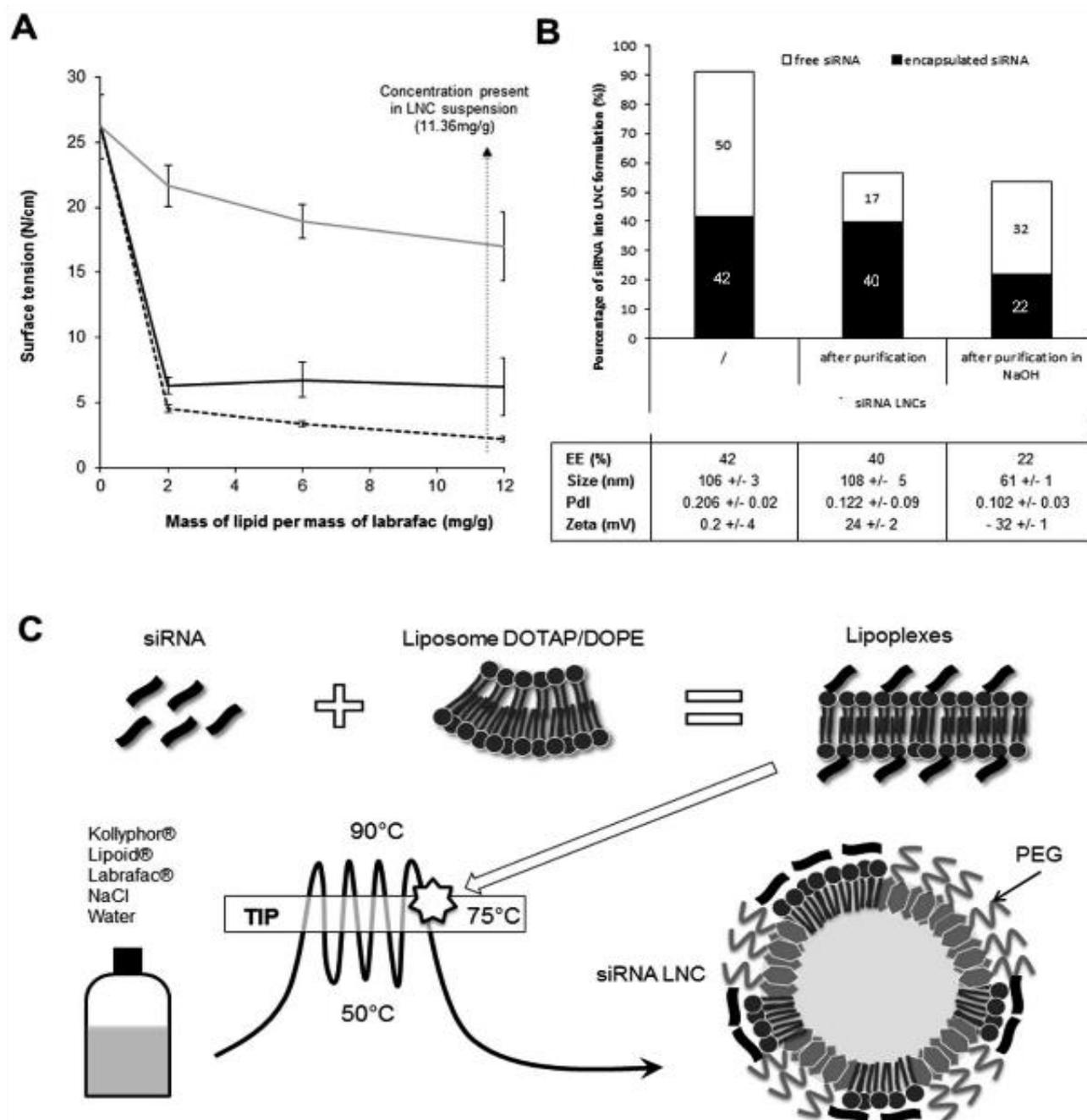
To explain LNC instability, surface tension studies were conducted at an oil-water interface. siRNA, liposomes, and lipoplexes were diluted in a water drop introduced into bulk Labrafac<sup>®</sup> oil. Application of siRNA alone only slightly modified the surface tension, even at the highest tested concentration, which can be attributed to the hydrophilic structure of the siRNA (Fig. 2A). DOTAP/DOPE lipids strongly reduced surface tension due to their amphiphilic structures, which feature polar heads and hydrophobic carbon chains. The lipoplexes had a stronger



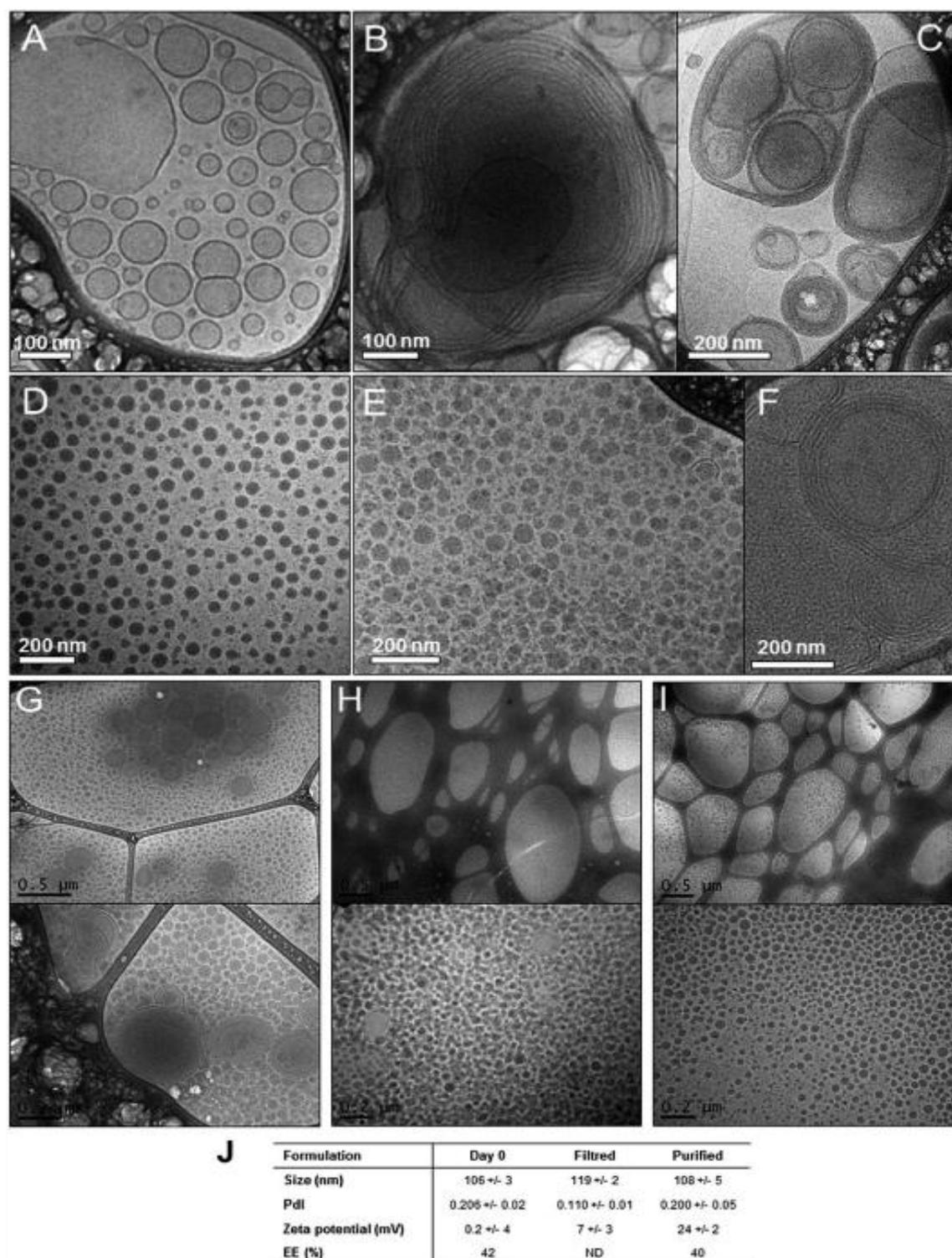
**Figure 1.** Size (—) and zeta potential (---) of siRNA LNCs over time for different (A) siRNA quantities, (B) charge ratios, (C) temperatures of lipoplex addition, (D) and storage temperatures. The encapsulation efficiency (EE) was determined by UV spectroscopy and EtB electrophoresis of (E) blank lipid nanocapsules (LNCs) over the course of 1 month, while (F) the other parameters were tested after 1 month of storage. ND: non-determined. Size and zeta measures were expressed as mean  $\pm$  standard deviation ( $n = 3$ ).

effect on surface tension than did the liposomes, confirming the interaction of lipoplexes with the water/oil interface. This phenomenon has also been described with another cationic lipid, EDOPC (*O*-ethyl-dioleoylphos-

phatidylcholine), in association with DNA plasmids. The importance of CR on interfacial properties has been demonstrated previously [29]. The tensio-active profile of EDOPC/DNA was obtained only at a high CR, i.e., 6,



**Figure 2** (A) Drop tensiometer analysis of DOTAP/DOPE liposomes (—), lipoplexes (---), and siRNA (---) at different concentrations of DOTAP/DOPE in Labrafac (mg/g). (B) Determination of siRNA encapsulation by UV spectroscopy and DLS-determined physico-chemical parameters of siRNA LNCs at day 0, after column purification, and after column purification in sodium hydroxide solution (0.15 M). (C) Process for siRNA LNC formulation by the phase inversion method, with schematic representation of liposome, lipoplexes, and siRNA LNC organization. Surface tension, size, and zeta measures were expressed as mean ± standard deviation ( $n = 3$ ).



**Figure 3.** Cryo-TEM imaging of siRNA LNC components: (A) DOTAP/DOPE liposomes at 30 mM+, (B, C) lipoplexes, (D) blank LNCs, (E) siRNA LNCs and (F) lipid microstructures in the siRNA LNC suspension. The siRNA LNC suspension was characterized (G) before filtration, (H) after filtration, and (I) after purification on a column. The corresponding physico-chemical characteristics are given in (J).

as opposed to 1, similar to that obtained here for DOTAP/DOPE lipoplexes at a CR of 5, suggesting their interfacial position.

The siRNA LNCs were purified by passage through a PD10 column in water and again in NaOH (0.15 M). NaOH was used to disrupt non-LNC-encapsulated lipoplexes potentially containing siRNA by destabilizing electrostatic interactions [16]. After purification in water, the size, zeta potential and EE of the siRNA LNCs were unchanged, while the PDI decreased by half. Taken together with the results of the UV assay, these results confirmed the removal of non-encapsulated siRNA. After purification with NaOH, the size of the siRNA LNCs decreased to 61 nm, while the surface charge became negative and similar to that of blank LNCs (Fig. 2B). In addition, the final encapsulation was estimated at 21%, versus 40% without NaOH (Fig. 2B). This result demonstrated that the encapsulated lipoplexes were sensitive to NaOH treatment, supporting lipoplex incorporation into the LNC shell. siRNA LNCs could be represented by a core-shell structure in which the tensio-active properties of the lipoplexes enable incorporation of siRNA into the shell, as has been reported previously for other siRNA delivery systems (Fig. 2C) [30–32].

### 3.2.2 Specific structure of siRNA LNCs observed by cryo-TEM

All components of the siRNA LNCs were examined by transmission electron microscopy using a cryogenic stage (cryo-TEM) with vitreous samples to ensure optimal preservation of the hydrated structures [19]. DOTAP/DOPE liposomes displayed the uni- or multilamellar structures classically observed for liposomes, with diameters ranging from 100 to 1000 nm (Fig. 3A) [33]. The siRNA lipoplexes exhibited a typical “onion-like” structure, with large sizes up to a micrometer, confirming the association of siRNA with cationic lipids via electrostatic interaction (Fig. 3B and C) [34]. The narrow size distribution and spherical structure of blank and siRNA LNCs were also observed (Fig. 3D and E). This microscopy study confirmed the size range of the siRNA and blank LNCs measured by DLS (approximately 100 and 50 nm, respectively). Lipid microstructures organized as multilamellar structures similar to the lipoplex edifices were also observed in non-purified suspensions of the siRNA LNCs (Fig. 3F). These structures might contain non-LNC-encapsulated siRNA, potentially explaining the observed EE of 40%.

### 3.2.3 Elimination of lipid microstructures by purification method

As suggested in our previous study into DNA LNCs [15], prior to *in vivo* administration the siRNA LNCs must be purified to eliminate non-encapsulated siRNA and, consequently, the lipid microstructures observed by cryo-TEM. Two purification methods were tested: (i) filtration at 0.2  $\mu\text{m}$ ; and (ii) purification with a Sephadex<sup>®</sup> column

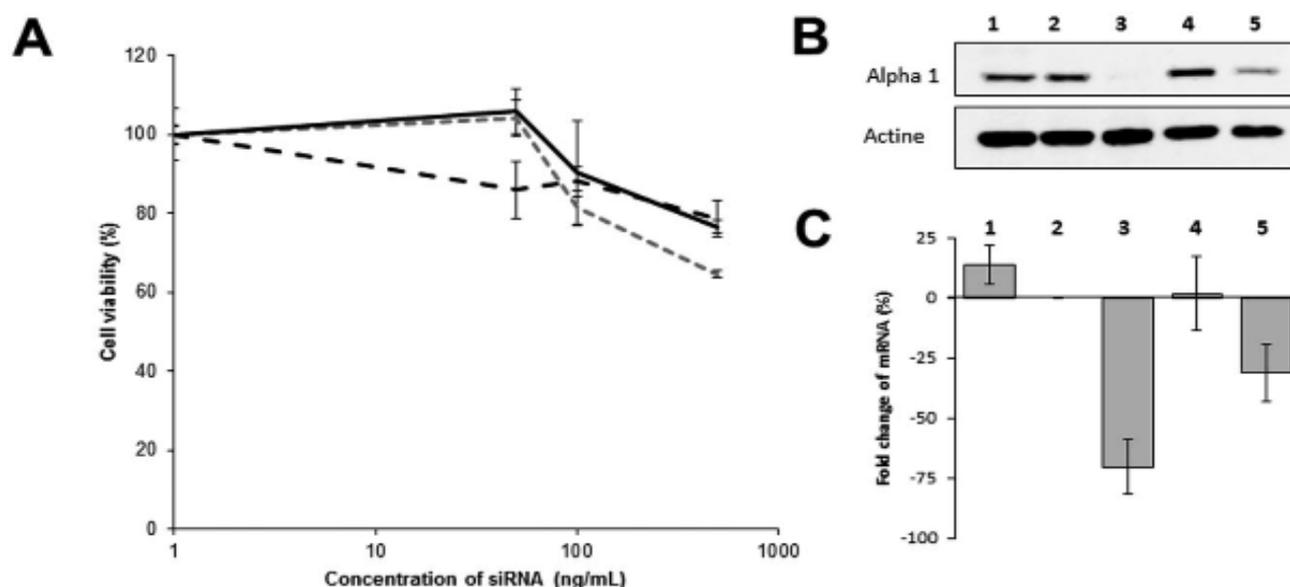
(Fig. 3H and I). For siRNA LNCs at day 0, we observed classical physico-chemical parameters with an EE of approximately 40% (Fig. 3J). As described previously, numerous lipid microstructures were observed in the LNC suspension by cryo-TEM (Fig. 3G). After filtration, no significant variation of the physico-chemical parameters was observed compared to the suspension at day 0, with the exception of a decrease in the PDI (Fig. 3H). At low blowback, analysis by cryo-TEM demonstrated that the large majority of lipid microstructures were eliminated (Fig. 3I). However, at high blowback, a few vesicles, smaller than 200 nm, passed through the filter and were observed in the LNC suspension. The presence of these vesicles is related to the extrusion method classically used to disrupt liposomes and obtain monodisperse populations [35]. After column purification, electron microscopy analysis revealed that the siRNA LNCs remained intact and the lipid microstructures had been removed. The column system appears to be the most effective method for the elimination of non-encapsulated siRNA retained in the lipid microstructures for the purification of siRNA LNC suspensions for *in vivo* experiments.

## 3.3 In vitro assays

### 3.3.1 Cytotoxicity evaluation and efficient transfection of melanoma cells

The alpha 1 subunit of the sodium pump NaK ATPase is an essential part of a protein complex allowing the active transport of sodium and potassium ions to regulate osmosis [36]. This protein is involved in cancerogenesis in melanoma and plays a part in the proliferation and survival of abnormal cell via the MAP kinase pathway. Moreover, the inhibition of this protein by chemical inhibitors has shown anticancerous effects with chemosensitivity [37]. However, the chemical inhibitors also appears to be strong cardiotoxic drugs. In this context, siRNA LNCs could represent a promising tool to specifically inhibit the alpha 1 subunit and enhance subsequent chemotherapy treatment of melanomas.

The effects of blank and siRNA LNCs on cell viability were evaluated on human melanoma SK-Mel28 cells. No cytotoxicity was observed after a 4 h incubation of siRNA LNCs with SK-Mel28, regardless of the kind of LNCs, even at a siRNA concentration of 500 ng/mL (equivalent to 32 nM), corresponding to a LNC concentration of 0.6 mg/mL (Fig. 4A). In contrast to chemical inhibitors, inhibition by alpha 1 siRNA alone at 3 days after 4 h transfection did not induce cell death in SK-Mel28 cells as shown in Fig. 4A [36]. Similarly, Karpova et al. observed no impact of alpha 1 siRNA on cell viability even four days after 72 h transfection of neuroblastoma cells [38]. siRNA efficacy was validated by comparison to the commercial transfection reagent Oligofectamine™ inducing total inhibition of the target protein (Fig. 4B and C). Protein and gene analysis of siRNA LNCs, at a low con-



**Figure 4.** Efficient inhibition of the alpha 1 subunit by siRNA LNCs without cytotoxicity on SK-Mel28. **(A)** Cytotoxicity was assessed using blank LNCs (—), 75°C siRNA LNCs (---), and 40°C siRNA LNCs (---) after evaluation of cell viability by MTS assay on the human SK-Mel28 melanoma cell line up to a concentration of 500 ng/mL LNCs. **(B)** Protein inhibition of the alpha 1 subunit was assessed using the positive control Oligofectamine™ LNCs. **(C)** Gene analysis by RT-q-PCR was performed to examine alpha 1 mRNA levels with Oligofectamine™ and LNCs. 1 = SK-Mel28 cells, 2 = Oligofectamine™ with control siRNA, 3 = Oligofectamine™ with alpha 1 siRNA, 4 = control siRNA LNCs, and 5 = alpha 1 siRNA LNCs. Viability and alpha 1 mRNA fold change were expressed as mean  $\pm$  standard error of the mean (SEM) ( $n = 3$ ).

centration corresponding to a 150 ng/mL, confirmed the efficacy of siRNA LNCs to induce partial inhibition of alpha 1 subunit after transfection of SK-Mel28 (30% of mRNA inhibition versus 70% with Oligofectamine™) (Fig. 4B and C).

Karpova et al. demonstrated the partial inhibition of mRNA with Lipofectamine™ reagent in neuroblastoma cells similarly to our result with LNCs. In their case, this inhibition was sufficient to obtain significant results by combining mRNA inhibition with antineoplastic drugs [38]. In literature, the transfection efficiency of nanoparticles usually ranges between 30% and total inhibition, for a variety of cell models and transfection protocols [4]. In future work, siRNA LNCs could be combined with dacarbazine or other anticancer drugs. If necessary, the amount of siRNA could be increased as described previously and in vivo protocols adapted for synergetic effects.

### 3.3.2 PEGylation step induces low complement protein consumption

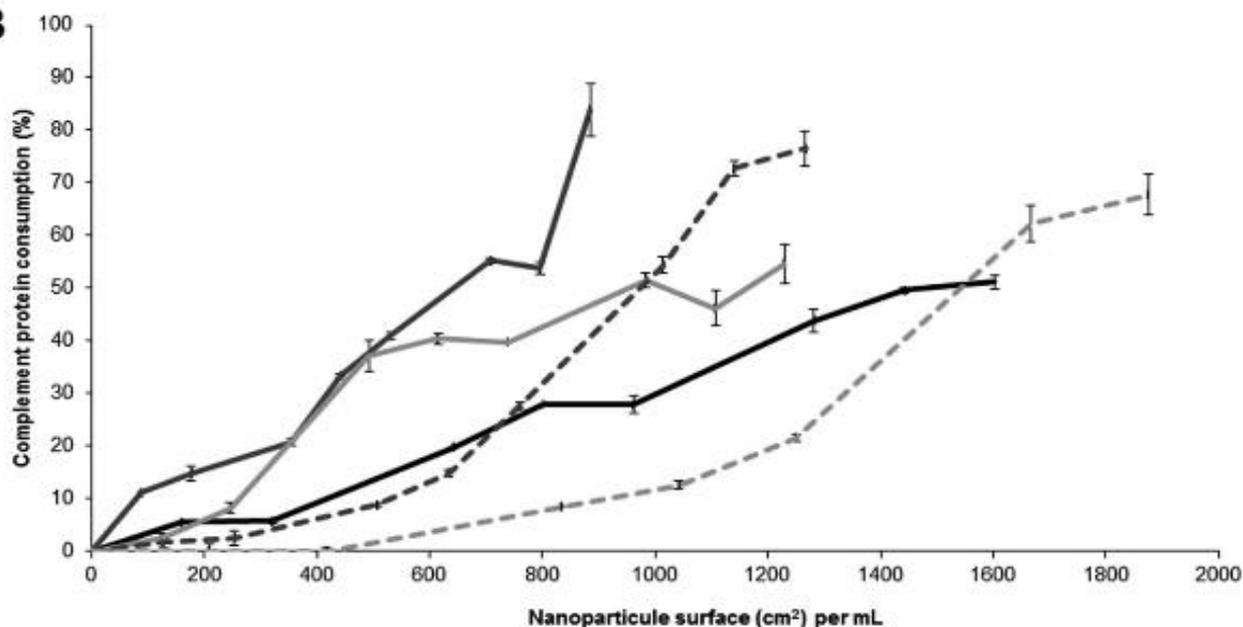
To enable a long circulation time in the blood, LNCs were modified by the addition of long PEG chains (2000 kDa) to create a hydrophilic ring around the LNCs by a post-insertion method described previously [12, 15]. Two formulations were tested, corresponding to lipoplex addition temperatures of 75°C and 40°C (Fig. 5A). After PEGylation, the surface charge decreased to approximately -14 mV for both siRNA LNC formulations due to the presence of the

negative PEG dipoles [39]. A higher PDI was observed after PEGylation due to the presence of free PEG chains in the LNC suspensions. Elimination of the free PEG chains by column purification reduced the PDI to less than 0.25, while maintaining the negative surface charge and adequate EE of the siRNA LNCs (Fig. 5A). Thus, this PEGylation method, which was initially developed for DNA LNCs, was found to also be suitable for siRNA LNCs.

To determine the efficacy of PEGylation, in vitro CH50 tests evaluating complement protein consumption were performed on blank, 75°C siRNA, and 40°C siRNA LNCs, as well as on the PEGylated form of both siRNA LNCs (Fig. 5B). No activation of the complement proteins was observed for siRNA alone (data not shown). The low activation profile of blank LNCs was previously ascribed to their low negative surface charge. Compared to blank LNCs, the positively charged surface of siRNA LNCs led to a greater consumption of complement proteins, with a higher response for 75°C siRNA LNCs. The negative charge of the PEGylated forms led to a reduced consumption of complement protein for the PEGylated siRNA LNCs compared to both non-PEGylated siRNA LNC formulations (75 and 40°C). These results confirm the integration of PEG on the siRNA LNC surface to produce “stealth” nanomedicines. The addition of PEG is a classic surface modification employed to improve the lifetime of nanomedicines in the blood [12, 40, 41]. In fact, the passive targeting of tumor sites via long-circulating drug

**A**

Formulation at 75°C	PEGylated	PEGylated and purified	Formulation at 40°C	PEGylated	PEGylated and purified
Size (nm)	104 +/- 2	73 +/- 4	Size (nm)	110 +/- 5	89 +/- 6
Pdl	0.293 +/- 0.10	0.167 +/- 0.02	Pdl	0.331 +/- 0.15	0.232 +/- 0.04
Zeta potential (mV)	-13 +/- 6	-12 +/- 7	Zeta potential (mV)	-14 +/- 3	-18 +/- 2
EE (%)	41	46	EE (%)	45	52

**B**

**Figure 5.** (A) Physico-chemical parameters of two PEGylated Lipid nanocapsule (LNC) formulations (75 and 40°C) before (left column) and after (right column) purification. (B) The activation profile of complement consumption was evaluated by CH50 assay of different formulations: blank LNCs (—○—), 75°C siRNA LNCs (—□—), 40°C siRNA LNCs (—△—), PEG 75°C siRNA LNCs (---○---), and PEG 40°C siRNA LNCs (---□---). Percentage values were expressed as mean ± standard error of the mean (n = 3).

delivery systems can be obtained by taking advantage of the enhanced permeability and retention (EPR) effect of PEGylation [42, 43].

#### 4 Concluding remarks

The development of nanomedicine carriers for systemic administration of siRNA is a major challenge. LNCs appear to be good candidates for siRNA delivery. This study confirmed the impact of the relative quantities of siRNA and lipid as well as storage conditions on the physico-chemical stability of siRNA LNCs. In addition, tensiometer analysis revealed the tensio-active properties of lipoplexes and supported the hypothesis of lipoplex shell localization. Cryo-TEM imaging validated the spherical structure of the siRNA LNCs as well as the removal, by column purification, of lipid microstructures formed

during the manufacture of the nanomedicine suspensions. No cytotoxicity against SK-Mel28 melanoma cells was observed at siRNA concentrations as high as 500 ng/mL. At a concentration under the threshold of toxicity, a promising decrease in alpha 1 protein was obtained with LNCs. Finally, the low complement activation of siRNA LNCs was demonstrated by CH50 assay. These results encourage the pursuit of *in vitro* and *in vivo* research to enable the application of this nanotechnology to cancer chemotherapy by co-encapsulating siRNA into the shell and a chemotherapeutic agent in the oily core of LNCs.

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The authors declare no financial or commercial conflict of interest.

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This issue of *Biotechnology Journal* is a focus issue on non-viral gene transfer and is edited by Tristan Montier, Chantal Pichon, Marc Blondel and Patrick Midoux. The cover was chosen with the intention of providing an essence of nucleic acid transfer technology. Image: DNA molecules © vitstudio Fotolia.com

## *Biotechnology Journal* – list of articles published in the November 2014 issue.

### Editorial: Current status and prospects on nucleic acid transfer

Tristan Montier, Chantal Pichon, Marc Blondel and Patrick Midoux

<http://dx.doi.org/10.1002/biot.201300510>

### Special Articles

#### Research Article

#### **Potato virus X-based expression vectors are stabilized for long-term production of proteins and larger inserts**

Christina Dickmeis, Rainer Fischer and Ulrich Commandeur

<http://dx.doi.org/10.1002/biot.201400347>

#### Research Article

#### **A robust transfection reagent for the transfection of CHO and HEK293 cells and production of recombinant proteins and lentiviral particles – PTG1**

Cristine Gonçalves, Fabian Gross, Philippe Guégan, Hervé Cheradame and Patrick Midoux

<http://dx.doi.org/10.1002/biot.201400324>

#### Research Article

#### **Efficient in vitro gene therapy with PEG siRNA lipid nanocapsules for passive targeting strategy in melanoma**

Pauline Resnier, Pierre LeQuinio, Nolwenn Lautram, Emilie André, Cédric Gaillard, Guillaume Bastiat, Jean-Pierre Benoit and Catherine Passirani

<http://dx.doi.org/10.1002/biot.201400162>

### Regular Articles

#### Biotech Method

#### **CRISPR/Cas9-mediated genome engineering: An adeno-associated viral (AAV) vector toolbox**

Elena Senís, Chronis Fatouros, Stefanie Große, Ellen Wiedtke, Dominik Niopek, Ann-Kristin Mueller, Kathleen Börner and Dirk Grimm

<http://dx.doi.org/10.1002/biot.201400046>

#### Research Article

#### **Hypoxia influences protein transport and epigenetic repression of CHO cell cultures in shake flasks**

Yueming Qian, Zizhuo Xing, Sherry Lee, Nancy A. Mackin, Aiqing He, Paul S. Kayne, Qin He, Nan-Xin Qian and Zheng Jian Li

<http://dx.doi.org/10.1002/biot.201400315>

#### Research Article

#### **Perfusion culture-induced template-assisted assembling of cell-laden microcarriers is a promising route for fabricating microtissues**

Xiu Wang, Qingling Jiao, Songjie Zhang, Zhaoyang Ye, Yan Zhou and Wen-Song Tan

<http://dx.doi.org/10.1002/biot.201400238>

#### Research Article

#### **Malaria vaccine candidate antigen targeting the pre-erythrocytic stage of *Plasmodium falciparum* produced at high level in plants**

Nadja Voepel, Alexander Boes, Güven Edgü, Veronique Beiss, Stephanie Kapelski, Andreas Reimann, Stefan Schillberg, Gabriele Pradel, Rolf Fendel, Matthias Scheuermayer, Holger Spiegel and Rainer Fischer

<http://dx.doi.org/10.1002/biot.201400350>

#### Research Article

#### **Validation of RetroPath, a computer-aided design tool for metabolic pathway engineering**

Tamás Fehér, Anne-Gaëlle Planson, Pablo Carbonell, Alfred Fernández-Castané, Ioana Grigoras, Ekaterina Dariy, Alain Perret and Jean-Loup Faulon

<http://dx.doi.org/10.1002/biot.201400055>

# **Curriculum Vitae**



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**PhD student in Biology and Nanotechnology**

### Work experience

*Since 2012: PhD student at Angers and Santiago of compostela, NanoFar student*

<u>Project:</u>	<b>Combination of nano and microcarriers for stem cell therapy of Huntington's disease: new regenerative medicine strategy (MINT, inserm u1066 at Angers)</b> with Associate Prof. Claudia MONTERO-MENEI and (Depart of Pharmaceutic and technology Pharmaceutical) Prof Alejandro SANCHEZ- BARREIRO
<u>New product development:</u>	Formulation of innovating synthetic vectors and microparticles Design of siRNA Development of GABAergic differentiation protocol
<u>New methods development and validation:</u>	<i>Cell culture:</i> culture of human Mesenchymal Stem Cells from bone marrow of patients, and differentiation into GABAergic neurons.  <i>Protein quantification:</i> Nuclear protein extraction, mastering the technique of Western blot and immunofluorescence.  <i>RNA quantification:</i> classic RT-qPCR to quantify mRNA (Design and validation of primers.) and quantification of RNA by agarose gel.  <i>SiRNA:</i> design and validation of SiRNA and transfection in the human cells.  <i>Formulation:</i> micro and nanoparticles formulation.

**2011: Internship at the University of Montpellier 1 at the Institute of Neuroscience in the team: "Mesenchymal Stem Cells, Environment and Articular Immunotherapy of Rheumatoid Arthritis" with PhD Danièle NOEL.**

<u>Project:</u>	<b>Identify the role of the micro-RNA during the chondrogenesis</b>
<u>New product development:</u>	Confirm the expression change of micro-RNA during the differentiation by RT-qPCR. Characterize the effect for one micro-ARN.
<u>New methods development and validation:</u>	<i>Cell culture:</i> Isolation and culture of human Mesenchymal Stem Cells from bone marrow of patients, and differentiation into chondrocyte.  <i>RT-qPCR:</i> Using an innovative protocol based on the poly(A) adenilation to determine the level of microRNAs expressed in a cell with SYBgreen and classic RT-qPCR to quantify ( design and validation of microRNA primers. LightCycler® 480).  <i>Transfection:</i> Characterization of the effect of a micro-RNA in human chondrogenic differentiation, thanks to its over-expression and its inhibition with commercial agents and electroporation.

**2010: Internship at the University of Montpellier 1 at the Institute for Research in Biotherapy, "Early Embryonic Development and Human Pluripotent Stem Cells" with PhD John DEVOS.**

*Subject: Introduction to research and study of pluripotency*

## Publication

- 2013: Characterization and structure of siRNA lipid nanocapsules for gene therapy" by Resnier, Pauline; LeQuinio, Pierre; Lautram, Nolwenn; André, Emilie; Gaillard, Cédric; Bastiat, Guillaume; Benoit, Jean-Pierre; Passirani, Catherine Article reference: NANO-101577

## Education

2015: **Animal experimentation:** conception and realization of animal protocol

2011: **Master's Degree in Biology-health**, University of Montpellier 1.  
Specialisation in "Genetic, epigenetic and control of cell determinism", 24<sup>th</sup> of 100.

## Skill

### **Scientific:**

- Development and Validation of new protocol.
- Practical knowledge in cell culture, transfection, immunofluorescence, RT-qPCR, western blot, UV spectrometrie and formulation.
- Theoretical knowledge of current techniques of biology.
- Operating results, writing analysis reports and analysis protocol.
- Bibliographic researches. Scientific articles, European Pharmacopeia.
- Maintenance of equipment.

### **Equipment:**

- Spectrophotometer:  
IRAffinity, Shimadzu ; UV-1607, Shimadzu.

### **Languages:**

- French: Native language.
- English: Intermediate.
- Spanish: Intermediate.

### **Informatic:**

- Word, Excel, Powerpoint.
- Zetasizer
- Shimadzu and Thermo control and software.

## Miscellaneous

### **Other :**

- vice president "solidarity and prevention" in the association *Ici,là-bas*
- pilote committee " financement and communication" for the Doctoriale 2014,

May 2013 - December 2013: Travel in Spain.

September 2010 - December 2011: Travel in France and Europe: Development of English.



# Thèse de Doctorat

Emilie ANDRE

Title Combination of nano and microcarriers for stem cell therapy of Huntington's disease: new regenerative medicine strategy

## Résumé

La combinaison de biomatériaux et cellules souches, a pour but de protéger des cellules endommagées et de ralentir la progression des maladies neurodégénératives, comme la maladie de Huntington (MH). Les cellules souches mésenchymateuses et particulièrement une sous-population, les cellules MIAMI, ont déjà démontré leur efficacité dans la maladie de Parkinson. Il est cependant essentiel d'améliorer leur différenciation neuronale, leur survie et évaluer leur sécrétome. L'objectif principal de ce travail fut de proposer une stratégie innovante de médecine régénératrice pour la MH associant cellules souches, nano et micro médecines. Pour l'évaluer, un nouveau modèle animale ex vivo de la MH a été mis en place. Nous avons ensuite développé et optimisé deux nano-vecteurs, des nanocapsules lipidiques et des nanoparticules solides de SPAN, et les avons associés à un inhibiteur de REST qui est un facteur de transcription qui empêche la différenciation neuronale. La transfection de ce siREST a montré une amélioration du phénotype neuronal. Ces cellules ainsi modifiées furent ensuite induites vers un phénotype GABAergic grâce à des facteurs de croissance. Puis elles ont été associées à un support 3D, les microcarriers pharmacologiquement actif (MPA) permettant une meilleure intégration des cellules après greffe. Les MPA sont des microsphères ayant une surface biomimétique de laminine et libérant de façon contrôlée un facteur trophique le « brain derived neurotrophic factor » (inducteur d'un phénotype neuronal et neuro-protecteur). Des résultats prometteurs ont été obtenus, encourageant à continuer l'évaluation de cette stratégie in vivo dans des modèles génétiques de la MH.

## Mots clés

**Médecine régénérative, cellules cellules souches mesenchymateuse, Maladie de Huntington, siREST, nanoparticules, microcarriers pharmacologiquement actif**

## Abstract

The combination of biomaterials and stem cells aims to protect damaged cells and slow the progression of neurodegenerative diseases such as Huntington's disease (HD). Mesenchymal stem cells, particularly a sub-population known as MIAMI cells, have already demonstrated their effectiveness in Parkinson's disease. However, it is essential to improve their neuronal differentiation, survival, and to assess their secretome. The main objective of this work was to propose an innovative regenerative medicine strategy for HD by combining stem cells, micro and nano medicines. To perform this assessment, a new ex vivo animal model of HD has been set up. We then developed and optimized two nanovectors, lipid nanocapsules and solid SPAN nanoparticles, carrying an inhibitor of REST a transcription factor, which prevents neuronal differentiation. The transfection of this siREST showed an improvement in the neuronal phenotype. These modified cells were then induced into a GABAergic phenotype through growth factors. They were then associated with a 3D support, the pharmacologically active microcarriers (PAM) allowing a high rate of engraftment. The PAM are microspheres which have a biomimetic surface of laminin and release a trophic factor BDNF, brain derived neurotrophic factor (inducer of a neural phenotype and neuroprotective) in a controlled manner. Promising results were obtained, further encouraging continuing the evaluation of this strategy in vivo in genetic models of HD.

## Key Words

**Regenerative medicine; mesenchymal stem cells, Huntington's disease, siREST, nanoparticles, pharmacologically active microcarriers.**