Cellules stromales mésenchymateuses et vecteurs polymériques pour l'ingénierie tissulaire du système nerveux central

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Affichée au dessus de mon bureau, depuis le premier jour de ma thèse, figurait cette phrase chaque jour un peu moins obscure :

« Comment utiliser les cellules MIAMI en combinaison avec les MPA pour permettre la survie et la différenciation in vivo ? »…

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TABLE DES MATIERES

ABBREVIATIONS

6-OHDA: 6-hydroxydopamine
AR: acide rétinoïque
AVC: accident vasculaire cérébral
BDNF: brain-derived neurotrophic factor
bFGF: basic fibroblast growth factor
BHA : butylated hydroxyanisole
BO: bulbe olfactif
BP: bleu de Prusse
BrdU: bromodeoxyuridine
COMT: catechol-O-methyl-transferase
CSE: cellule souche embryonnaire
CSM: cellule stromale mésenchymateuse
CSN: cellule souche neurale
DAT: dopamine transporter
DMSO: dimethylsulfoxide
EGF: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
ES: embryonic stem (cell)
FDA: food and drug administration
FN: fibronectine
FGF8b: fibroblast growth factor 8b
g-CSF: granulocyte-colony stimulating factor
GDNF: glial cell line-derived neurotrophic factor
GFP: green fluorescent protein
GMP: good manufacturing practices
HEDP: 1-hydroxyethylidene-1.1-bisphosphonic acid
HGF: hepatocyte growth factor
HPLC: high pressure liquid chromatography
hRPE: human retinal pigment epithelium
ICF: immunocytofluorescence
IF: immunofluorescence
IHC: immunohistochimie
iPS: induced pluripotent stem (cell)
IRM: imagerie par résonance magnétique
ITS: insulin-transferrin-selenium
IV: intra-veineuse
LM : laminine
MAO : monoamine oxidase
MEC : matrice extra-cellulaire
MIAMI: marrow-isolated adult multilineage inducible
MPA: microcarriers pharmacologiquement actifs
NGF: nerve growth factor
NT3: neurotrophine 3
PDL: poly-D-Lysine
PEG: polyéthylène glycol
PLGA: acide poly(lactique-co-glycolique)
RT-qPCR: real-time quantitative polymerase chain reaction
SHH: sonic hedgehog
SNC: système nerveux central
SPIO: superparamagnetic iron oxide
SVF: sérum de veau fœtal
TGF: transforming growth factor
TH: tyrosine hydroxylase
VEGF: vascular endothelial growth factor
ZSV: zone sous-ventriculaire

INTRODUCTION GENERALE

La thérapie cellulaire consiste en la greffe de cellules dans le but de prévenir, traiter ou atténuer une maladie. Ce domaine, encore en plein essor, est toutefois déjà très développé avec certains types de cellules. Citons la transplantation de cellules souches hématopoïétiques utilisée pour le traitement des maladies hématologiques (Korbling and Fliedner 1996; Rocha, Chastang et al. 1998; Nagatoshi, Kawano et al. 2002), les greffes de peau (Kuroyanagi, Kubo et al. 2004), les recherches menées pour la réparation osseuse ainsi que les veines, foies et vessies artificielles (Atala 2001; Mahler, Desille et al. 2003; Grellier, Granja et al. 2009; Konig, McAllister et al. 2009). Cependant, pour les organes avec un niveau de complexité supérieur, le cerveau en étant l'archétype, la thérapie cellulaire a encore de nombreux défis à relever.

De nombreuses recherches en thérapie cellulaire visent actuellement à trouver des stratégies permettant de soigner les patients atteints de pathologies cérébrales telles que les accidents vasculaires cérébraux (AVC), les traumatismes crâniens ou encore les maladies neurodégénératives. Les AVC sont la troisième cause de mortalité dans le monde (environ 150 000 personnes aux Etats-Unis par an) et les traumatismes crâniens constituent un autre problème de santé majeur avec plus d'1,4 million de cas par an aux Etats-Unis (Langlois, Kegler et al. 2003). Dans ces deux affections, les séquelles sont lourdes, avec un coût matériel et humain important. Il faut noter que seules des mesures de prévention primaires, comme la prise d'aspirine et une bonne hygiène de vie, ou secondaires, comme la thrombolyse, sont disponibles dans le cas des AVC. Aucune approche de neuroprotection ou de neuroréparation n'a fait ses preuves pour l'instant, que ce soit pour les traumatismes crâniens ou les AVC. Notons que la mobilisation des cellules souches hématopoïétiques par injection de « granulocyte-colony stimulating factor » (g-CSF), technique couramment utilisée pour le traitement des maladies hématopoïétiques, est actuellement étudiée pour ses éventuels effets neuroprotecteurs dans le cas des AVC (England 2009).

Le nombre de personnes atteintes chaque année par les maladies neurodégénératives est en augmentation constante, principalement en raison d'une espérance de vie de plus en plus élevée. De même que pour les AVC et les traumatismes crâniens, aucun traitement efficace n'est disponible à ce jour afin de protéger ou de réparer le tissu cérébral lésé dans le cas de la maladie d'Alzheimer, d'Huntington ou de Parkinson, d'où l'intérêt de développer des alternatives telles que la thérapie cellulaire. En raison de leurs progressions lentes, les maladies neurodégénératives constituent des modèles idéaux pour la thérapie cellulaire à visée neuroprotectrice mais également neuroréparatrice. Ce travail de thèse traitera plus particulièrement de la maladie de Parkinson, la plus étudiée des maladies neurodégénératives

en raison de la zone relativement restreinte du tissu cérébral lésé, mais également de la spécificité du type cellulaire touché par la maladie (Olanow and Tatton 1999; Lindvall and Kokaia 2009).

James Parkinson décrit en 1817 la maladie qui porte son nom et qui touche environ 2 % de la population de plus de 65 ans, avec plus d'un million de cas rapportés en 2006 dans l'Union Européenne (Cohen 2006; Kim and de Vellis 2009). On observe chez les patients parkinsoniens une dégénérescence neuronale et notamment des fibres dopaminergiques provenant de la substance noire et innervant le striatum (noyaux caudés et putamen chez l'Homme), aboutissant à une déplétion en dopamine au niveau du striatum. Bien que d'autres zones puissent être touchées par la maladie, cette dégénérescence du faisceau dit « nigro-strié » est principalement responsable de divers problèmes moteurs tels la rigidité, les tremblements, l'akinésie ainsi que la perte de réflexes posturaux. Les neurones dopaminergiques qui dégénèrent possèdent souvent des corps d'inclusion appelés Corps de Lewy, constitués d'accumulation de protéines, principalement de l'α-Synucleine (Spillantini, Schmidt et al. 1997). Les causes de cette pathologie sont encore assez méconnues, hormis de rares cas d'origine génétique (Brassat, Durr et al. 1999; Spacey and Wood 1999; Lesage and Brice 2009). Plusieurs facteurs sont cependant couramment suggérés, comme la mort cellulaire programmée, l'augmentation du stress oxydatif, des dysfonctions mitochondriales, des dommages excitotoxiques, des infections virales, ainsi qu'une toxicité environnementale (Olanow and Tatton 1999; Hatcher, Pennell et al. 2008).

Comme pour les autres troubles cérébraux cités précédemment, aucun traitement n'est actuellement totalement satisfaisant pour la maladie de Parkinson. Le plus utilisé, la prise de L-DOPA, précurseur de la dopamine, permet d'en augmenter la synthèse par les neurones survivants mais cause également de nombreux effets secondaires sur le long terme (Papa, Engber et al. 1994; Fahn 1996). La L-DOPA est d'ailleurs souvent combinée avec de la carbidopa (Duodopa ®) afin d'éviter le métabolisme périphérique de synthèse de la dopamine, et de l'entacapone (Stalevo ®), un inhibiteur de la catechol-O-methyl transferase (COMT) afin de prolonger la durée d'action de la dopamine synthétisée (figure 1). Des traitements chirurgicaux, tels que la pallidotomie et la thalamotomie, sont également parfois proposés mais restent risqués et traumatisants pour le patient (Koller, Pahwa et al. 1999; Walter and Vitek 2004). Une importante diminution des symptômes de la maladie peut être obtenue grâce à la stimulation électrique profonde, le coût prohibitif de cette technique en étant cependant le principal inconvénient. Notons toutefois qu'une très récente étude clinique décrit un possible ralentissement de la progression de la maladie lors de la prise précoce de Rasagiline, un

médicament habituellement destiné à atténuer les symptômes de la maladie par son effet inhibiteur sur la « monoamine oxidase » (MAO) (Olanow, Rascol et al. 2009).

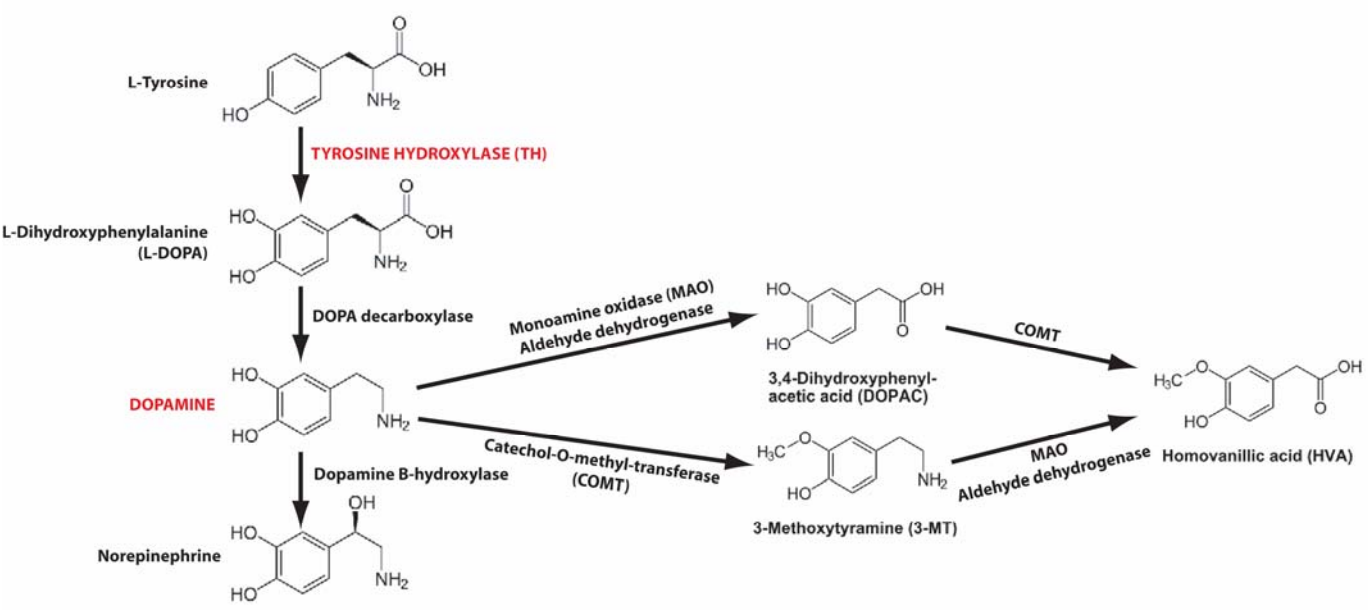

Figure 1. Voies de synthèse et de dégradation de la dopamine

Le précurseur de la dopamine, la L-DOPA est synthétisé à partir de L-Tyrosine par la tyrosine hydroxylase (TH), enzyme clef du métabolisme dopaminergique. La présence de TH est souvent utilisée pour caractériser les neurones de phénotype dopaminergique. La norépinéphrine (ou noradrénaline) peut être synthétisée à partir de dopamine, cette dernière pouvant être dégradée par 2 enzymes, la « monoamine oxidase » (MAO) et la « catechol-O-methyl-transferase » (COMT).

C'est pour toutes ces raisons que la thérapie cellulaire apparaît comme une alternative attirante pour le traitement de la maladie de Parkinson, les cellules transplantées pouvant permettre de restaurer les fonctions perdues lors de la dégénérescence des fibres dopaminergiques endogènes. En effet, la transplantation de cellules productrices de dopamine au niveau du striatum, ou encore de cellules neuronales se différenciant en cellules de phénotype dopaminergique, pourrait permettre de restaurer un taux normal de dopamine dans cette zone du cerveau, bien que cette stratégie ne permette pas de restaurer les connexions substance noire-striatum du faisceau nigro-strié (figure 2). De plus, selon le type cellulaire choisi, les cellules transplantées peuvent aussi avoir un effet protecteur voire réparateur sur les fibres dopaminergiques survivantes, grâce à la production de facteurs de croissance et de chimiokines.

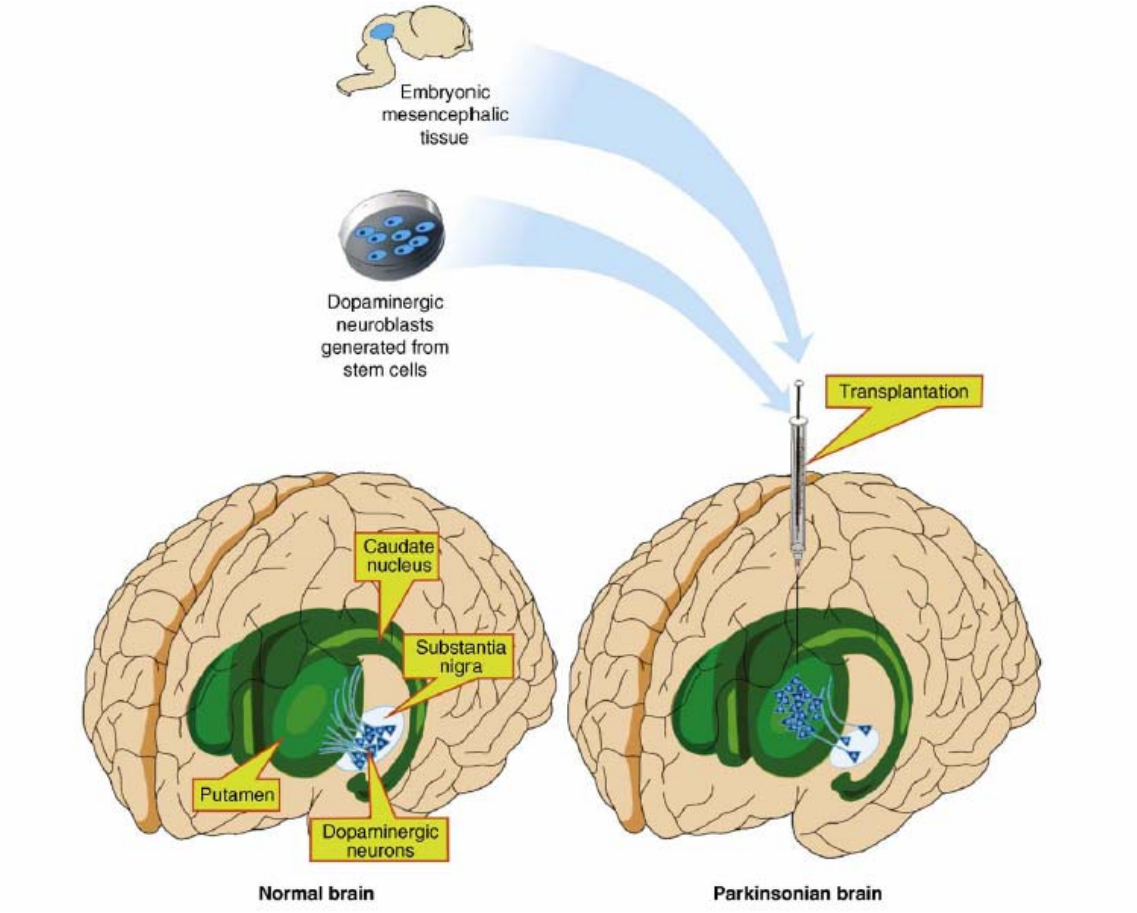

Figure 2. Thérapie cellulaire pour la maladie de Parkinson

Dans le cerveau sain, les neurones dopaminergiques de la substance noire innervent le striatum (putamen et noyaux caudées) où ils secrètent de la dopamine. Dans le cas de la maladie de Parkinson, la dégénérescence de ces fibres dopaminergiques aboutit à une déplétion striatale en dopamine. La majorité des approches de thérapie cellulaire visent à transplanter des cellules productrices de dopamine dans le striatum afin de restaurer un taux normal de dopamine, sans toutefois permettre de restaurer les connexions substance noire-striatum (figure reproduite de Lindvall et Kokaia, Cell, 2009).

De nombreux essais de transplantation de tissus ou de cellules dissociées ont été réalisés chez l'Homme pour la thérapie cellulaire de la maladie de Parkinson. Les premiers essais cliniques réalisés par transplantation autologue de cellules chromaffines adultes de la glande médullo-surrénale, en raison de leur capacité à produire de la dopamine (Drucker-Colin and Verdugo-Diaz 2004; Fernandez-Espejo, Armengol et al. 2005), ont permis d'observer des bénéfices fonctionnels pendant 10 mois chez de jeunes patients parkinsoniens (Madrazo, Drucker-Colin et al. 1987). Cependant, les bénéfices provenaient plus probablement d'un effet trophique des cellules transplantées, car seule une faible fraction des cellules (1 %) était véritablement capable de synthétiser de la dopamine. De plus, très peu de

cellules étaient détectées 1 à 2 ans après transplantation, remettant en cause les effets à long terme de cette approche (Hurtig, Joyce et al. 1989). Les cellules de l'épithélium pigmenté de la rétine (« human retinal pigment epithelium », hRPE) sont un autre type de cellules utilisé pour leur capacité à produire le précurseur de la dopamine, la L-DOPA (Stover and Watts 2008). Ces cellules, obtenues à partir de cultures cellulaires provenant de banques d'organes humains, peuvent être amplifiées et stockées pendant un temps prolongé, rendant ainsi aisé leur utilisation en thérapie cellulaire. Cependant, leur origine post-mortem les expose à des critiques d'ordre éthique. En dépit de l'expression de la « tyrosine hydroxylase » (TH), enzyme clef permettant la conversion de la L-DOPA en dopamine, ces cellules ne produisent que très peu de dopamine (Pawelek and Korner 1982). Après transplantation, leur capacité à produire de la L-DOPA *in situ* peut toutefois permettre de diminuer, voire supprimer, la prise orale de ce précurseur de la dopamine.

Les cellules embryonnaires et fœtales issues du mésencéphale ventral, contenant une fraction de précurseurs dopaminergiques, ont ensuite été largement étudiées pour la thérapie de la maladie de Parkinson (Freed, Breeze et al. 1992; Widner, Tetrud et al. 1992). Malgré les bénéfices fonctionnels obtenus, le plus souvent estimés par le test « unified Parkinson's disease rating scale » (UPDRS), d'importants problèmes de standardisation des cellules implantées ainsi que des controverses éthiques s'opposent à cette stratégie. De plus, leur origine cause également d'énormes problèmes logistiques pour une application clinique à grande échelle, plusieurs fœtus étant nécessaires pour un unique patient.

L'utilisation de cellules souches, c'est-à-dire de cellules possédant d'importantes capacités d'auto-renouvellement et de différenciation, permet de surmonter ce problème de disponibilité, en fournissant une source de cellules potentiellement illimitée pour la thérapie cellulaire. Ainsi, plusieurs études ont démontré l'intérêt des cellules souches embryonnaires et des cellules souches nerveuses (CSN) d'origine fœtale pour la thérapie cellulaire dans des modèles animaux de la maladie de Parkinson (Lindvall and Kokaia 2009). Les cellules souches embryonnaires ont l'avantage d'être pluripotentes, possédant ainsi un large potentiel de différenciation vers les 3 feuillets embryonnaires (endo, méso et ectoderme). Les CSN peuvent quant à elles être source de neurones, mais également d'astrocytes et d'oligodendrocytes à la fois *in vitro* et *in vivo* (Lois and Alvarez-Buylla 1993; Garcia-Verdugo, Doetsch et al. 1998; Lindvall, Kokaia et al. 2004). Ces cellules, peuvent donc fournir une source virtuellement illimitée de précurseurs de neurones dopaminergiques en dépit des problèmes éthiques liés à leur origine.

Les cellules souches issues d'individus adultes, comme par exemple les cellules stromales mésenchymateuses (CSM) mais également les CSN adultes, ne sont quant à elles soumises à aucun problème d'ordre éthique et offrent en plus la possibilité de pouvoir réaliser des greffes autologues sans causer de tératomes. Les CSN adultes sont principalement localisées dans le gyrus denté de l'hippocampe, la zone sous-ventriculaire (ZSV) et le bulbe olfactif (Pagano, Impagnatiello et al. 2000; Roy, Wang et al. 2000; Zhao, Momma et al. 2003) et présentent l'avantage d'être déjà engagées vers un phénotype neuronal. La ZSV striatale et corticale, et plus particulièrement la bordure du ventricule latéral, est à l'origine d'un important flux migratoire de CSN appelé courant de migration rostral. Ces CSN, ou neuroblastes, ont la capacité de migrer vers le bulbe olfactif où elles se différencient alors en cellules neuronales post-mitotiques (Coskun and Luskin 2002; Menezes, Marins et al. 2002; Taupin and Gage 2002). Pour des applications thérapeutiques, le prélèvement de CSN chez l'Homme est laborieux et risqué en raison de leur localisation, bien qu'une étude récente décrive le prélèvement avec succès de CSN chez un patient parkinsonien adulte, avec un gain fonctionnel significatif après réimplantation des cellules mises en culture (Arias-Carrion and Yuan 2009; Levesque 2009).

Contrairement aux CSN adultes, les CSM ont l'avantage de pouvoir être prélevées très aisément par ponction de moelle osseuse. Ces cellules, découvertes grâce aux travaux de A. J. Friedenstein à partir de la fin des années 60 (Friedenstein, Petrakova et al. 1968) et isolées de la moelle osseuse par leur capacité d'adhérence au plastique, constituent un élément important de la niche hématopoiétique *in vivo*. Depuis ces études, des cellules aux propriétés de CSM ont été décrites dans virtuellement tous les organes du corps humain, et semblent constituer une composante de l'environnement périvasculaire (Sensebe, Krampera et al. 2009). Les CSM sont une population hétérogène de cellules, constituée de cellules souches mais également de précurseurs déjà engagés dans une voie de différenciation. On préférera donc désormais l'appellation « cellules stromales mésenchymateuses » à l'appellation « cellules souches mésenchymateuses », terme utilisé dans le chapitre 2 (Horwitz, Le Blanc et al. 2005).

D'aspect fibroblastique après mise en culture, les CSM possèdent la capacité de se différencier en cellules des différents phénotypes mésodermaux, notamment en ostéoblastes, chondrocytes, adipocytes, cellules stromales de la moelle osseuse, ainsi qu'en cellules endothéliales et musculaires. Sous certaines conditions, ces cellules semblent également capables de se différencier en cellules d'origine non-mésodermique telles qu'en cellules épithéliales (Sensebe, Krampera et al. 2009). Particulièrement intéressant pour la thérapie cellulaire du système nerveux central (SNC), de plus en plus d'études décrivent la

différenciation de CSM en cellules présentant des caractéristiques neuronales à l'aide de divers protocoles *in vitro*, un processus que l'on qualifiera simplement de « différenciation neuronale » dans la suite de ce travail de thèse (Song and Sanchez-Ramos 2003; Hermann, Maisel et al. 2006; Ross and Verfaillie 2008). Cette capacité de différenciation neuronale est également parfois décrite *in vivo,* comme par exemple lors de protocoles de greffe de moelle osseuse chez l'Homme (Mezey, Chandross et al. 2000; Mezey, Key et al. 2003), bien que la fraction de cellules différenciées reste toujours faible *in vivo* après transplantation dans différents modèles animaux (Kopen, Prockop et al. 1999; Li, Chen et al. 2002; Zhao, Duan et al. 2002; Jendelova, Herynek et al. 2004; Esneault, Pacary et al. 2008). Ces cellules semblent également douées de capacités immuno-modulatrices, de par les spécificités de leur complexes majeurs d'histocompatibilité, mais peut-être plus encore par la sécrétion de molécules pouvant affecter la réponse du système immunitaire (Le Blanc 2003; Le Blanc, Tammik et al. 2003; Gotherstrom, Ringden et al. 2004; Maitra, Szekely et al. 2004; Nasef, Mathieu et al. 2007; Rossignol, Boyer et al. 2009; Sensebe, Krampera et al. 2009). Un effet anti-inflammatoire pouvant protéger les neurones dopaminergiques a également été décrit *in vivo, via* l'inactivation de la microglie par les CSM (Kim, Park et al. 2008). Enfin, ces cellules possèdent une forte capacité à migrer, principalement vers des sites présentant des lésions.

Dans le SNC, la migration des CSM vers des lésions d'origines ischémiques, tumorales, neurotoxiques ou encore purement mécaniques a été décrite dans le cerveau adulte (Mahmood, Lu et al. 2002; Jendelova, Herynek et al. 2004; Hellmann, Panet et al. 2006; Sykova and Jendelova 2007a; Delcroix, Jacquart et al. 2009; Sadan, Bahat-Stromza et al. 2009). Il semblerait que cette capacité de migration soit causée par la production de molécules inflammatoires et de chimiokines par le tissu lésé (Ozaki, Nishimura et al. 2007; Spaeth, Klopp et al. 2008). Par exemple, des molécules chimioattractantes, comme la « monocyte chemoattractant protein-1 » (MCP-1), la « macrophage inflammatory protein 1α » (MIP-1α) et l'interleukine (IL-8) (Wang, Li et al. 2002), produites par le tissu cérébral dans un modèle animal d'AVC, semblent responsables de cette migration par le biais d'interactions avec leurs récepteurs membranaires. Une étude décrit également la migration de CSM implantées dans des cerveaux sains de souris, jeunes et adultes, une capacité liée à l'expression de récepteurs (Neo1, Nrp2, Robo1 et 4) connus pour réguler la migration des cellules neuronales (Phinney, Baddoo et al. 2006).

En raison des multiples avantages procurés par les CSM, ce travail de thèse s'est concentré sur l'utilisation de ces cellules pour la thérapie cellulaire du SNC, et plus particulièrement sur une sous-population humaine de CSM, les cellules « marrow-isolated adult multilineage inducible » (MIAMI) (D'Ippolito, Diabira et al. 2004). Les cellules MIAMI, obtenues de la moelle osseuse de vertèbres ou de la crête iliaque, ont la particularité d'être isolées en préservant des conditions similaires à leur niche *in vivo*, notamment à 3 % d'O_2, et sont ensuite amplifiées en faible densité (procédé breveté). Elles expriment de nombreux marqueurs de cellules souches pluripotentes, dont l'important trio de facteurs de transcription Oct4, Sox2 et Nanog, mais également une variété de facteurs de croissance et de chimiokines ainsi que le récepteur à l'« hepatocyte growth factor » (HGF) (D'Ippolito, Howard et al. 2006). Ce dernier est notamment connu pour améliorer le potentiel de migration et de régénération tissulaire des CSM (Rosova, Dao et al. 2008). De plus, les cellules MIAMI peuvent se différencier en cellules des trois feuillets embryonnaires, y compris en cellules de phénotype neuronal. Notons que la neurotrophine 3 (NT3) est essentielle à la survie et à la différenciation neuronale des cellules MIAMI *in vitro* au cours d'un protocole de différenciation en 3 étapes réalisé sur un substrat de fibronectine (FN), une molécule de la matrice extra-cellulaire (MEC). Ce protocole, dont le but est de mimer la différenciation des CSN (Tatard, D'Ippolito et al. 2007) permet d'obtenir 50 à 60 % de cellules MIAMI présentant des caractéristiques de cellules neuronales immatures avec un potentiel de membrane similaire à celui des neurones, mais toutefois incapables de générer des potentiels d'actions. De plus, les cellules MIAMI, comme d'autres CSM, peuvent être induites *in vitro* en cellules de phénotype dopaminergique exprimant la TH, enzyme clef de la biosynthèse de dopamine (Tatard, D'Ippolito et al. 2007; Trzaska, Kuzhikandathil et al. 2007; Barzilay, Kan et al. 2008).

Des études *in vivo* ont récemment montré les bénéfices de CSM humaines dans divers modèles murins de la maladie de Parkinson, avec pour origine principale la sécrétion de facteurs de croissance et de chimiokines par les CSM (Bouchez, Sensebe et al. 2008; McCoy, Martinez et al. 2008; Sadan, Bahat-Stromza et al. 2009). Une étude décrit toutefois une expression de la TH par les CSM transplantées dans un modèle de lésion totale de la maladie de Parkinson, l'effet bénéfique observé provenant alors vraisemblablement d'une réelle différenciation neuronale des CSM *in situ* (Levy, Bahat-Stroomza et al. 2008). Les CSM ont également prouvé leurs intérêts dans des modèles animaux d'AVC et de traumatismes crâniens (voir pour revue (Li and Chopp 2009)), probablement en raison de leur capacité à produire une variété de facteurs de croissance et de chimiokines. De plus, un essai clinique où

les CSM furent transplantées en intraveineuse (IV) chez des patients souffrants d'AVC semble démontrer la sûreté d'utilisation de CSM autologues chez l'Homme (Bang, Lee et al. 2005).

Quel que soit le type de cellules transplantées, leurs faibles survie et intégration dans le tissu hôte constitue le problème majeur des thérapies visant le SNC. Dans le cadre de la maladie de Parkinson, la mort rapide des cellules après implantation est probablement due à leur nécrose suite au stress mécanique encouru lors de leur préparation ou implantation, mais également à leur apoptose à posteriori (Brundin, Karlsson et al. 2000; Isacson, Bjorklund et al. 2003; Olanow, Schapira et al. 2003). En effet, malgré les bénéfices fonctionnels observés lors de tests UPDRS chez des patients parkinsoniens, une forte mort cellulaire est pratiquement toujours décrite, avec seulement 10 à 20 % des cellules greffées qui survivent 24 heures après implantation (Brundin, Barbin et al. 1985; Kordower, Rosenstein et al. 1996; Kordower, Freeman et al. 1998; Schierle, Hansson et al. 1999).

L'ingénierie tissulaire s'impose désormais comme une stratégie d'avenir pour régénérer efficacement un tissu lésé. Le principe de l'ingénierie tissulaire repose sur l'utilisation de biomatériaux, ou « scaffolds », fournissant un support adéquat en 3 dimensions aux cellules transplantées. Ces biomatériaux peuvent améliorer le comportement des cellules *in vivo*, notamment en terme de survie et de différenciation, comme cela a été démontré dans différents modèles de pathologies du SNC (Tatard, Menei et al. 2005; Orive, Anitua et al. 2009). Afin de pouvoir être implantés au sein du SNC, ces « scaffolds » doivent idéalement être de petite taille et constitués de biomatériaux biodégradables et biocompatibles. Ainsi, des microsphères de verres ou de Cytodex ® (des microsphères polysaccharidiques enrobées de collagène, une molécule de la MEC) ont permis d'améliorer la survie ainsi que le gain fonctionnel de cellules chromaffines dans un modèle murin de la maladie (Cherksey, Sapirstein et al. 1996; Borlongan, Saporta et al. 1998). De façon similaire, la survie de cellules hRPE est nettement augmentée par la combinaison avec des microsphères biocompatibles de gélatine dans des modèles murins et de primates non-humains, le tout sans avoir recours à une immunosuppression. Ce concept prometteur, nommé Spheramine ® (pour revue, voir (Stover and Watts 2008)) est même actuellement en phase II d'essai clinique multicentrique (en double-aveugle, randomisé & contre placebo).

De plus, l'utilisation de composants biomimétiques associés aux « scaffolds » peuvent être utilisés afin de guider avantageusement la survie, la prolifération mais également la différenciation des cellules vers un phénotype donné, par exemple neuronal. Les effets de ces molécules biomimétiques, le plus souvent dérivés de la MEC, sur la différenciation neuronale,

ont initialement été décrits avec des cellules neuronales fœtales et des lignées cellulaires comme les cellules PC12, alors que la plupart des études faites avec les CSM se concentraient sur leur différenciation vers un phénotype ostéogénique ou chondrogénique (Docheva, Popov et al. 2007). Désormais, quelques études décrivent l'effet de molécules dérivées de la MEC sur les CSM dans un contexte neuronal. Citons par exemple l'utilisation de gels de collagène, procurant un support favorable à l'adhésion de CSM dans un modèle animal de traumatisme crânien et permettant de réduire le volume de la lésion cérébrale, ce qui n'est pas observé lors de la transplantation de cellules seules (Lu, Mahmood et al. 2007).

Les microcarriers pharmacologiquement actifs (MPA) (Tatard, Venier-Julienne et al. 2005) sont un outil d'ingénierie tissulaire mettant à profit cette stratégie biomimétique en combinaison avec la libération d'un facteur de croissance au sein d'un vecteur unique et simple. La libération contrôlée de protéines thérapeutiques encapsulées au sein de microsphères d'acide poly(lactique-co-glycolique) (PLGA), polymère approuvé par la « food and drug administration » (FDA), est une thématique largement développée dans notre laboratoire (Giteau, Venier-Julienne et al. 2008a; Giteau, Venier-Julienne et al. 2008b). Le principal défi technologique de cette stratégie consiste à obtenir une libération prolongée de la protéine sous une forme biologiquement active. Le « glial-derived nerve growth factor » (GDNF) a, par exemple, déjà été encapsulé avec succès, et son efficacité démontrée *in vivo* dans un modèle murin de la maladie de Parkinson (Jollivet, Aubert-Pouessel et al. 2004; Garbayo, Montero-Menei et al. 2009). Dans ces études, la libération prolongée du GDNF actif par les microsphères de PLGA a été confirmée au cours de cinétiques de libération *in vitro* par dosage de la protéine (ELISA et radioactivité) ainsi que par un bioessai permettant de vérifier son activité. Les MPA sont des microsphères de PLGA biodégradables et biocompatibles recouvertes d'une molécule de la MEC permettant l'adhésion des cellules et fournissant un microenvironnement adéquat en 3 dimensions *in vitro* et *in vivo*. De plus, les MPA libèrent un facteur thérapeutique au cours de leur dégradation, qui peut agir en synergie avec la surface biomimétique pour maintenir la survie et favoriser la différenciation des cellules transportées, mais qui pourra également avoir un effet direct sur le tissu hôte (Tatard, Venier-Julienne et al. 2005). Après implantation, la dégradation des MPA est totale en 2-3 mois environ, et permet donc une intégration optimale des cellules dans le parenchyme cérébral sans induire de réaction inflammatoire notoire (Tatard, Venier-Julienne et al. 2004; Tatard, Venier-Julienne et al. 2005; Tatard, D'Ippolito et al. 2007). De part le choix de la molécule utilisée pour la surface biomimétique et du facteur encapsulé, les MPA constituent un outil multifonction utilisable dans de nombreux contextes et avec toute une variété de cellules (figure 3).

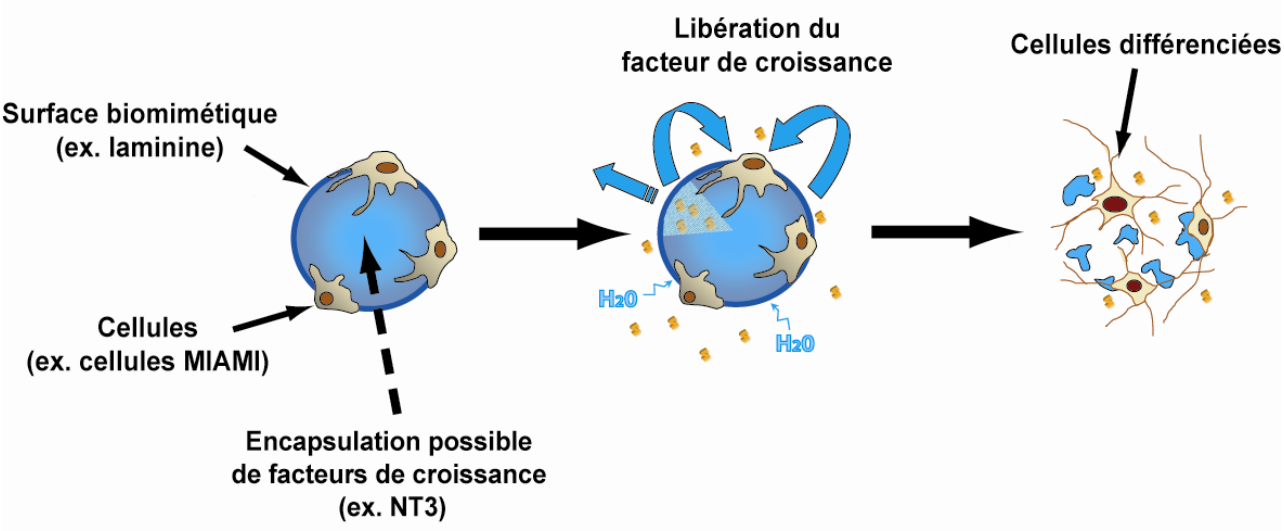

Figure 3. Concept des microcarriers pharmacologiquement actifs

Des microsphères de PLGA, polymère biodégradable et biocompatible, sont enrobées d'une surface biomimétique constituée, par exemple, d'une protéine de la matrice extracellulaire (MEC) comme la laminine, afin de permettre l'adhésion de cellules, telles les cellules MIAMI. Cette protéine de la MEC, en plus de favoriser leur adhésion, peut également permettre de maintenir ou d'orienter la différenciation des cellules transportées. Un facteur de croissance pourra éventuellement être encapsulé au sein du PLGA. Celui-ci, libéré de façon contrôlée lors de la dégradation des microsphères, pourra agir en synergie avec la surface biomimétique pour favoriser la survie et la différenciation des cellules transportées, mais pourra également avoir une action directe sur le tissu hôte. Enfin, après dégradation totale des microsphères, les cellules peuvent intégrer facilement le tissu hôte.

Les preuves du concept des MPA ont tout d'abord été apportées avec une lignée de cellules dérivées de la médullo-surrénale, les cellules PC12, qui possèdent la capacité de se différencier et de produire de la dopamine en réponse à une exposition au « nerve growth factor » (NGF) lorsqu'elles adhèrent à un substrat. La transplantation de ces cellules, en combinaison avec des MPA recouverts de FN et libérant du NGF dans un modèle de rats hémi-parkinsoniens, a permis d'améliorer significativement la survie et la différenciation des cellules PC12, en corrélation avec une baisse de leur prolifération. De plus, un effet comportemental bénéfique a été observé chez les rats greffés avec ces complexes (Tatard, Venier-Julienne et al. 2004). L'intérêt du concept des MPA fut ensuite démontré de façon similaire avec des précurseurs dopaminergiques issus du mésencéphale ventral attachés à des MPA libérant du GDNF, qui induit la survie et la différenciation de ces neurones. Après implantation dans un modèle de rats hémi-parkinsoniens, nous avons constaté que les MPA permettaient d'augmenter la survie des cellules embryonnaires véhiculées, mais également la

densité de fibres dopaminergiques, ce qui se traduit par une amélioration comportementale par rapport aux rats traités avec des cellules embryonnaires seules. L'intérêt de ce travail résidait aussi dans la faible quantité de cellules nécessaire pour observer ces effets (Tatard, Sindji et al. 2007).

Objectifs du travail de thèse:

La mise en œuvre d'une stratégie d'ingénierie tissulaire pour la réparation du SNC soulève de nombreuses questions, comme par exemple le choix du type cellulaire qui procurera les plus importants bénéfices, avec le moins de contraintes à la fois pratiques mais également éthiques. Les cellules adultes semblent ainsi idéales en raison de la réalisation possible de greffes autologues, tout en évitant les problèmes éthiques rencontrés avec les cellules d'origines fœtale ou embryonnaire. Les CSM semblent tout particulièrement adaptées à cette application en vertu de leur potentiel de différenciation neuronale, ainsi que de leur capacité à produire tout un panel de facteurs de croissance, pouvant avoir des effets neuroprotecteurs et/ou neuroréparateurs mais également immuno-modulateurs.

Le domaine de l'ingénierie tissulaire est encore peu exploré en combinaison avec des cellules adultes, alors que ce type de stratégies devrait pouvoir permettre d'améliorer leur comportement *in vivo*. Par conséquent, une revue bibliographique, ayant pour objectif de poser les bases de la contribution possible de l'ingénierie tissulaire pour la thérapie cellulaire du cerveau à l'aide de cellules adultes, fait l'objet du **chapitre 1** de cette thèse :

L'ingénierie tissulaire peut-elle améliorer la thérapie cellulaire du cerveau à l'aide de cellules adultes ? (Revue bibliographique)

Dans le but de combiner CSM et ingénierie tissulaire pour la réparation du cerveau, nous nous sommes tout d'abord questionnés sur la migration possible des CSM, ainsi que sur leur survie et éventuelle différenciation neuronale, après implantation dans le cerveau de rat. D'où la première question directrice de ce travail de thèse, développée dans le c**hapitre 2**:

Quel est le devenir des cellules stromales mésenchymateuses *in vivo*?

Expérimentalement, nous avons donc choisi d'étudier le devenir des CSM après implantation par stéréotaxie dans la ZSV en bordure du ventricule latéral, une zone hautement neurogénique et favorable à la migration cellulaire. En effet, c'est de cette zone que proviennent les CSN qui migrent ensuite en suivant le courant de migration rostral pour atteindre finalement le bulbe olfactif (Coskun and Luskin 2002; Menezes, Marins et al. 2002). Afin d'observer la migration des cellules transplantées au cours du temps, nous avons utilisé une méthode de suivi non-invasive d'imagerie par résonance magnétique (IRM), permettant de visualiser les cellules ayant préalablement incorporé des nanoparticules d'oxyde de fer enrobées de bisphosphonates (Portet, Denizot et al. 2001).

Dans un second temps, nous avons tenté d'améliorer le potentiel de différenciation neuronale d'une sous-population de CSM humaines, les cellules MIAMI, au cours de leur expansion *in vitro* par une méthode simple consistant en un pré-traitement en « epidermal growth factor » (EGF) et « basic fibroblast growth factor » (bFGF), des mitogènes couramment utilisés pour l'expansion de CSN *in vitro*. Ainsi, la seconde question directrice de ce travail de thèse, développée dans le **chapitre 3,** était :

Est-il possible d'améliorer simplement la différenciation neuronale de cellules stromales mésenchymateuses humaines avec un pré-traitement en facteurs de croissance lors de leur expansion ?

L'EGF et le bFGF sont en effet souvent utilisés pour la différenciation neuronale de CSM *in vitro*, mais également parfois avant leur implantation pour la thérapie cellulaire du SNC. Cependant, les changements induits par ces facteurs ne sont que très peu décrits dans la littérature et nous nous sommes donc efforcés de caractériser précisément leurs effets sur le potentiel de différenciation neuronale des cellules MIAMI *in vitro*. Une amélioration de ce potentiel neurogénique devait ensuite nous permettre d'obtenir une meilleure intégration et plus de bénéfices fonctionnels après transplantation dans le cadre de notre stratégie d'ingénierie tissulaire pour la thérapie du SNC.

Cette stratégie consistait en l'utilisation de MPA, optimisés afin de permettre leur utilisation avec les cellules MIAMI pré-traitées en EGF-bFGF, pour la thérapie cellulaire du SNC dans le contexte de la maladie de Parkinson. D'où la dernière question directrice de ce travail de thèse, développée dans le **chapitre 4** :

Les microcarriers pharmacologiquement actifs (MPA), adaptés pour une utilisation avec les cellules stromales mésenchymateuses humaines, peuvent-ils améliorer le comportement de rats hémi-parkinsoniens ?

Dans le but d'améliorer davantage la survie, la différenciation neuronale et la capacité de réparation des cellules MIAMI *in vivo,* des MPAs disposant d'une surface biomimétique de laminine (LM), une protéine de la MEC, furent mis au point après avoir caractérisé *in vitro* les effets de cette molécule sur la différenciation neuronale des cellules MIAMI. Nous avons finalement évalué l'effet de greffes de cellules MIAMI combinées à des MPA-LM dans un modèle de rats hémi-parkinsoniens lésés à la 6-hydroxydopamine (6-OHDA).

CHAPITRE 1

Revue bibliographique

L'ingénierie tissulaire peut-elle améliorer la thérapie cellulaire du cerveau à l'aide de cellules adultes ?

Adult cell therapy for brain neuronal damages: Where do we stand with tissue engineering?

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ABSTRACT

For most brain neurological disorders, including stroke, traumatic brain injury and neurodegenerative disorders, no long term and effective treatments are currently available. Therefore, pursuing novel approaches in cell therapy, a highly promising strategy, is not only desired but essential. However, alternatives to embryonic and fœtal cell sources have to be found in order to overcome major ethical, as well as tissue availability and rejection concerns.

In this regard, adult cells, mainly mesenchymal stromal cells, would allow an easy isolation from the patient body, therefore permitting autologous grafts to be performed. Here, we describe the current use of adult cells for brain therapy, with a special emphasis on mesenchymal stromal cells, adult neural stem cells, as well as adrenal chromaffin and retinal pigment epithelium cells. However, the major problems of cell survival and engraftment, as well as control of differentiation and cell fate after transplantation, are still encountered with the use of adult cells.

Tissue engineering, providing a 3-dimensional support to grafted cells, dramatically improves cell survival. Continuing developments in this field have led to emerging concepts such as the biomimetic approach, where the combined use of scaffolds with extracellular matrix molecules may improve the control of cell proliferation, survival, migration, differentiation and engraftment *in vivo*. Therefore, we later discuss the major scaffold properties to take into account for brain cell therapy as well as develop and illustrate the major advances in the tissue engineering field that may be implemented in combination with adult cells for brain therapy. Finally, we describe a novel approach developed in our laboratory to repair/protect lesioned tissues: the pharmacologically active microcarriers.

Keywords: brain disorders, mesenchymal stromal cells, adult neural stem cells, adrenal chromaffin cells, retinal pigment epithelium cells, scaffolds, biomimetic surface, pharmacologically active microcarriers.

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1. INTRODUCTION

The field of cell therapy is under constant expansion and already offers successful therapeutic strategies for some patients. Skin grafts, with organised cell banking systems (Kuroyanagi, Kubo et al. 2004), is one of the best examples where cell therapy provides clinically applicable results which already treats many patients. However, for organs with a higher level of complexity such as the brain, the focus in this review, cell therapy remains a challenging task. Nevertheless, the lack of long-term effective treatments for brain neuronal disorders such as stroke, traumatic brain injury (TBI) and neurodegenerative diseases, underlines the need to find alternative therapies to restore lost functions.

Stroke is the third leading cause of death in the world, and most patients are left dependent on others. About 150,000 Americans die of stroke every year and clinical management, with the exception of aspirin medication, thrombolytic therapy and hemicraniectomy, is only based on a supportive care. TBI is another major health problem, with over 1.4 million new cases reported annually in the USA (Langlois, Kegler et al. 2003). Similarly to stroke, TBI causes severe disability and there is still no clinical treatment available worldwide to repair the damaged tissue. The number of people affected by neurodegenerative diseases every year consistently increases, as a result of a higher life expectancy associated with poor environmental conditions. Parkinson's disease (PD) is the most studied neurodegenerative disorder and typically affects 2 % of the population after 65 years old, with more than 1 million cases reported in the European Union in 2006 (Cohen 2006; Kim and de Vellis 2009). PD is characterized by rigidity, tremor, akinesia and loss of postural reflexes. These symptoms are mainly due to an extensive loss of dopaminergic neurons in the substantia nigra pars compacta and their terminals in the striatum, therefore leading to a striatal deficiency of dopamine. Although other regions may be affected, the localized dopaminergic neurodegeneration makes PD an ideal target for cell therapy. The aetiology of idiopathic PD is not known but the dopaminergic depletion may be attributed to programmed cell death of neurons containing protein aggregates called Lewy bodies (Spillantini, Schmidt et al. 1997), viral infection, or environmental insults (Hatcher, Pennell et al. 2008). In some rare cases, a genetic component has been observed in some families, see for review (Brassat, Durr et al. 1999; Spacey and Wood 1999; Lesage and Brice 2009). The current treatment for PD is the oral administration of L-DOPA, a precursor of dopamine, but its long-term administration has been known for quite some time to produce severe detrimental side effects (Papa, Engber et al. 1994; Fahn 1996). Moreover, the continuing

degeneration of dopaminergic neurons renders the treatment less effective. Surgical procedures, such as deep brain stimulation, pallidotomy and thalamotomy are either too onerous or too traumatic for an extensive development (Koller, Pahwa et al. 1999; Walter and Vitek 2004). Huntington's disease (HD), the second most studied neurodegenerative disorder, is caused by polyglutamate expansions in the huntingtin protein (exon 1 of chromosome 4) and is therefore a heritable disorder. HD results in neuronal dysfunction and degeneration, mainly of medium spiny striatal GABAergic neurons. Neuronal degeneration contributes to progressive physiological, motor, cognitive, and emotional disturbances, such as dementia and choreic symptoms. As no cure is currently available, management of symptoms is, in the same manner as for PD, the primary goal in treating HD.

All these facts illustrate the compelling need to develop new therapeutic approaches for these disorders. Brain cell therapy may be one of these strategies and could allow the functional replacement of missing or damaged neurons by transplanting cells that may differentiate into the desired phenotype and integrate the host parenchyma, or alternatively rescue the affected neuronal population, resulting in the production of growth factors and missing neurotransmitters that will promote recovery. Working with various animal models of neurological disorders, several teams have tried to prevent the loss of neurons or to replace them, using mainly neuronal precursors and lately, since the discovery of stem cells, embryonic stem (ES) cell, induced pluripotent stem (iPS) cells, or neural stem cell (NSC) grafts. All of these studies demonstrate the potential of cell therapy to repair the injured brain. However, this approach still faces many problems related to cell survival, control of cell fate (e.g. avoidance of teratoma formation), maintenance of a defined differentiated phenotype and proper cell engraftment after transplantation. Cell survival is one of the most challenging technical issues as only a small percentage (10-20 %) of implanted cells survive 2 weeks after transplantation in the context of PD (Brundin, Barbin et al. 1985; Schierle, Hansson et al. 1999; Brundin, Karlsson et al. 2000; Isacson, Bjorklund et al. 2003; Olanow, Schapira et al. 2003). In addition, using cells from embryonic or foetal origins raises major ethical as well as availability concerns. For all these reasons, the identification of alternative sources of cells is of great importance.

In this review, we will report brain cell therapy studies performed with adult non transformed cells that, by nature, allow the use of autologous tissues for transplantation and overcome the immunological, availability, as well as ethical concerns (table 1 and 2). Moreover, these adult cells may be less prone to favour tumour or teratoma progression than, for example, ES cells or iPS cells. Experimental studies of adult cell therapy of the brain

primarily investigated the potential of adrenal chromaffin cells, which may produce dopamine, for the treatment of PD. These studies led to many clinical trials that reported modest improvements in some of the patients (Drucker-Colin and Verdugo-Diaz 2004). More recently, human retinal pigment epithelium (hRPE) cells were used as a "dopamine producing factory" for the treatment of PD, both in animal models and in humans (Stover and Watts 2008). Interestingly, the survival after transplantation of both these cell types has been increased via tissue engineering approaches (Cherksey, Sapirstein et al. 1996; Borlongan, Saporta et al. 1998; Stover and Watts 2008), which we will later discuss in this review.

Some adult stem cells have been studied for brain cell therapy, due to their self renewal and extensive differentiation potentials as well as ease of isolation from adult donors. These are hematopoietic stem cells (HSCs, i.e. CD34+ cells), present in bone marrow, and more importantly multipotent mesenchymal stromal cells (MSCs), primarily described in bone marrow as HSCs supporting cells. MSCs, a mixed cell population including stem and progenitor cells, are also often referred to as mesenchymal stem cells. However, this term should now be reserved for a subset of these cells that demonstrate stem cell activity by clearly stated criteria (Horwitz, Le Blanc et al. 2005). This review will focus on MSCs, due to their neuronal protection/repair potential, while HSCs preferentially differentiate into macro- and microglia in the brain (Eglitis and Mezey 1997). MSCs may be easily isolated from the iliac crest and expanded as a result of their self-renewal capacity, thus allowing autologous grafts to be performed. In addition, due to their large differentiation potential and their immunoregulatory properties (Le Blanc 2003; Le Blanc, Tammik et al. 2003; Gotherstrom, Ringden et al. 2004; Maitra, Szekely et al. 2004; Nasef, Mathieu et al. 2007), MSCs hold great promises for cell therapy. Indeed, these multipotent stem cells may mainly give rise to immature neuronal cells (Song and Sanchez-Ramos 2003; Hermann, Maisel et al. 2006; Tatard, D'Ippolito et al. 2007; Ross and Verfaillie 2008)(Delcroix, Curtis et al. 2009, under review) and a fraction of them may respond to microenvironmental cues and differentiate into neural-like cells after transplantation into adult rat brains (Kopen, Prockop et al. 1999; Zhao, Duan et al. 2002; Jendelova, Herynek et al. 2004). Therefore, even if efforts remain to be done to better understand and improve their neuronal differentiation both *in vitro* and *in vivo*, MSCs may become a valuable tool for brain neuronal cell therapy. In addition, MSCs that can migrate towards sources of lesions in the brain (Mahmood, Lu et al. 2002; Jendelova, Herynek et al. 2004; Hellmann, Panet et al. 2006; Sykova and Jendelova 2007a; Delcroix, Jacquart et al. 2009), may also provide a functional improvement in animal models, either directly or indirectly by their ability to produce various growth factors or other paracrine

factors that may mediate tissue repair or maintenance (Chen, Li et al. 2002; Li, Chen et al. 2002; Mahmood, Lu et al. 2002; Zhang, Li et al. 2005; Ohtaki, Ylostalo et al. 2008)(Garbayo, Curtis et al. manuscript in preparation). Finally, their administration in the central nervous system is feasible and seems to be safe in human subjects (Bang, Lee et al. 2005). For all these reasons, MSCs may become a clinically viable technology for cell therapy of the central nervous system that is not hindered by ethical and tissue rejection-related concerns.

Adult NSCs, that are already committed to the neuronal lineage, may give rise to neurons, astrocytes and oligodendrocytes both *in vitro* and *in vivo* (Lois and Alvarez-Buylla 1993; Garcia-Verdugo, Doetsch et al. 1998; Lindvall, Kokaia et al. 2004). These cells are mainly localized in the dentate gyrus of the hippocampus, the subventricular zone (SVZ) and the olfactory bulb (Pagano, Impagnatiello et al. 2000; Roy, Wang et al. 2000; Zhao, Momma et al. 2003). Adult NSCs still remain challenging to harvest, therefore explaining their limited use. However, a recent cell therapy clinical study describe the successful isolation of adult NSCs from a Parkinsonian patient; with autologous grafts resulting in functional benefits for the patient (Arias-Carrion and Yuan 2009).

To overcome the poor cell survival and engrafment 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* (reviewed in (Tatard, Menei et al. 2005; Orive, Anitua et al. 2009)). However, delivery of cells with scaffolds to the damaged brain still remains challenging due to practical limitations of delivery (Bible, Chau et al. 2009). The focus of this review is to provide an overview to the reader of what solutions tissue engineering may provide for adult cell therapy of the brain. Therefore, in the first section, we will review the major advances in the field of cell therapy using adult cells for the treatment of stroke, TBI and neurodegenerative disorders. In a second section, we will review advances in the tissue engineering field to improve brain cell therapy as well as the past and current *in vivo* studies using such approaches in combination with adult cells.

2. ADULT CELL THERAPY FOR BRAIN NEURONAL DAMAGES

2.1. Adult stem cells for stroke and traumatic brain injury therapy

MSCs are the most widely investigated adult stem cells for brain cell therapy in the context of various disorders and are, to our knowledge, the only adult cell type studied for stroke and TBI treatment (table 1). Most cell therapy studies performed with bone marrow MSCs resulted in improved outcome and cognitive function in experimental animal models of stroke, such as middle cerebral artery occlusion (MCAO) (Li and Chopp 2009) and transient common carotid artery occlusion (tCCAO) (Ohtaki, Ylostalo et al. 2008). Functional improvements are observed whatever the implantation route, intravenously or intracerebrally, even if reduction in infarct volume is not always observed (Li, Chopp et al. 2000). MSCs have the potential to migrate toward the lesioned brain, even after intravenous injection (Chen, Li et al. 2001). Migration may occur via the production in the damaged brain of chemokines such as stromal-derived factor 1 (SDF1), which may bind its receptor CXCR4 present on MSCs. Some studies described bone marrow cells recruitment and differentiation into NeuN positive cells in mice or human brains, in the context of marrow or MSC transplantation (Mezey, Chandross et al. 2000; Mezey, Key et al. 2003). Similarly, in ischemic rat models, systemic administration of MSCs, sometimes in combination with erytropoeitin (EPO), improved neurogenesis and functional recovery, with the detection of MSCs expressing neuronal or glial marker in the brain (Li, Chen et al. 2002; Esneault, Pacary et al. 2008). However, in these studies, cell survival was low and the number of differentiated cells very small, so that differentiation of transplanted MSCs into neuronal cells is not considered as the major recovery mechanism in the context of stroke (England 2009). Neurological benefits are often described after MSC injections and are assumed to mainly derive from the increased production of growth factors and other paracrine factors from MSCs in the ischemic tissue. Noteworthy, factors secreted by the ischemic brain itself, such as vascular endothelial growth factor (VEGF) and EPO, are also thought to play a major role in brain protection from ischemia (Tang, Pacary et al. 2006). MSCs combined with EPO (Esneault, Pacary et al. 2008), or alone but at a higher dose (Chen, Li et al. 2003), may reduce apoptosis in the penumbral zone of the lesion and favour the proliferation of endogenous cells in the subventricular zone (Li, Chen et al. 2002). In this regard, a recent study described the implication of Notch pathway in the latter mechanism (Wang, Chopp et al. 2008). In addition, MSCs have been described to reduce the thickness of the scar walls (Li, Chen et al. 2005) and may also favour angiogenesis (Chen, Zhang et al. 2003; Chopp, Li et al. 2008) as well as synaptogenesis

(Chen and Chopp 2006). MSCs transplantation may thus contribute to patient recovery by neurorepair and neuroprotection mechanisms via a reduction of apoptosis and the stimulation of host repair responses during acute and chronic stroke. Modulation of inflammatory and immune response or production of neuroprotective chemokines such as fractalkine and monocyte chemoattractant protein-1 (MCP-1) by MSCs are others mechanisms that may be involved in neuronal protection during ischemia (Re and Przedborski 2006; Ohtaki, Ylostalo et al. 2008; Madrigal, Leza et al. 2009)(Garbayo, Curtis et al. manuscript in preparation).

Cells	Origins	Recipients	Scaffolds	Benefits	Reference
MSC	Mouse	MCAO mouse model		Functional benefits but no reduction in infarct volume	Li, Chopp et al. 2000
	Rat	MCAO rat model		IV transplantation: migration toward the lesion	Chen, Li et al. 2001
	Human	MCAO rat model		IV transplantation: importance of growth factor increase in the ischemic tissue	Li, Chen et al. 2002
	Rat	MCAO rat model		IV transplantation: enhanced proliferation of endogeneous precursors	Chen, Li et al. 2003
	Rat	MCAO rat model		IV transplantation in combination with EPO: functional recovery and neurogenesis A fraction of MSCs with neuronal and glial markers	Esneault, Pacary et al. 2008
	Rat	MCAO rat model		IV transplantation: reduced thickness of the scar walls	Li, Chen et al. 2005
	Human	MCAO rat model		IV transplantation: promotion of angiogenesis	Chen, Zhang et al. 2003
	Human	tCCAO mouse model		Modulation of inflammatory and immune responses	Ohtaki, Ylostalo et al. 2008
	Human	ACA rat model	FN-coated PLGA microspheres	Neuroprotection of CA1 hippocampal neurons with MIAMI cells: neuroprotection enhanced with microspheres	Garbayo, Curtis et al. Manuscript under preparation
	Human	TBI rat model	Collagen cylinder	Scaffolds improved spatial learning, sensorimotor function & reduced the lesion volume	Lu, Mahmood et al. 2007
	Human	TBI rat model	Collagen cylinder	Delayed transplantation: increased angiogenesis in the injured cortex & transcallosal fiber length	Xiong, Qu et al. 2009
	Human	TBI mouse model	Collagen cylinder	Scaffolds improved spatial learning, reduced lesion volume & increased vascular density	Qu, Xiong et al. 2009
	Human	Human (autologous, stroke)		Proof of safety for MSCs use in human	Bang, Lee et al. 2005
NSC (teeth)	Human	MCAO rat model		Functional benefits	Yang, Chen et al. 2009

Table 1. Adult cell therapies for stroke and traumatic brain injury

Abbreviations: ACA: asphyxial cardiac arrest; EPO: erythropoietin; FN: fibronectin; IV: intravenous; MCAO: middle cerebral artery occlusion; MIAMI: marrow isolated adult multilineage inducible; MSC: mesenchymal stem cell; NSC: neural stem cell; tCCAO: transient common carotid artery occlusion, UPDRS: unified Parkinson's disease rating scale.

Similar therapeutic strategies are investigated for TBI using MSCs, which may also improve functional benefits whatever the injection route. However, a maximum of 10 % implanted MSCs may transdifferentiate into neuronal cells *in vivo* so that this mechanism was assumed not to be solely responsible for the functional benefits observed in TBI animal models (Li and Chopp 2009). Growth factor production by MSCs (Insulin-like growth factor (IGF1), VEGF, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and

epidermal growth factor (EGF)) stimulated by the lesioned brain (Chen, Katakowski et al. 2002), may be one of the main mechanisms leading to functional recovery by promoting glial, neuronal and blood vascular remodelling (Li and Chopp 2009). Notably, hippocampal precursors may be involved in tissue regeneration after TBI (Richardson, Sun et al. 2007). However, despite the benefits obtained using this approach, no significant changes in the lesion volume were observed, a problem that tissue engineering alternatives may resolve (see section 3.2.2).

In the context of stroke and TBI, very few studies were performed with adult NSCs, but it is interesting to mention an original study in a rat middle cerebral artery occlusion (MCAO) model with adult NSCs isolated from human wisdom teeth that survive within the brain and led to significant functional improvements (Yang, Chen et al. 2009). To conclude, there is still no large scale clinical trial underway for stroke and TBI therapy with adult cells. However, Bang et al. published a clinical trial report (Bang, Lee et al. 2005) that underwent criticisms so that it was thereafter considered as a study allowing to minimize the safety concerns on the clinical use of MSCs (De Keyser 2005).

2.2. Adult cells for neurodegenerative disorder therapy

Most of the cell therapy studies for neurodegenerative disorders have focused on Huntington's and Parkinson's disease (table 2), mainly with embryonic or foetal cells (Lindvall and Kokaia 2009). Alzheimer's disease (AD) is not an ideal target for cell therapy due to the multiple sites of the brain affected in this disease. Therefore, even if there is an increasing number of adult cell therapy studies for AD (Chen, Boiteau et al. 2006; Wu, Li et al. 2007; Lee, Jin et al. 2009), we will not focus on this disorder as no tissue engineering strategies have yet been described.

2.2.1. Adult cells

The earliest transplantation study in an animal model of HD appeared in 1983 and led to moderate functional benefits after transplantation of foetal rat striatal tissue fragments (Deckel, Robinson et al. 1983). Cell therapy is now becoming increasingly investigated, with many studies focusing on foetal tissue, ES cells or NSC grafts, mostly in quinolinic acid (QA)-induced animal model of the disease (Lee, Chu et al. 2005; Clelland, Barker et al. 2008; Kim, Lee et al. 2008). Several clinical trials have been performed with foetal-derived cells since the 1990s with quite successful results, but cell therapy for HD is still not widely available due to ethical, logistical or safety concerns (Kelly, Dunnett et al. 2009). Concerning adult cells, we can only mention early studies made with Sertoli cells which investigated their

protective effects when grafted into the 3-nitropropionic acid (3-NP) animal model of HD, effects that may result from Sertoli cells trophic and anti-inflammatory potentials (Emerich 2004).

One of the first cell therapy strategies to treat PD has been performed with adult cell types synthesizing dopamine or its precursor L-DOPA, in order to replace the degenerating neurons and to replenish the striatum with dopamine. These studies focused on the use of cells from the sympathoadrenal lineage, such as adult chromaffin cells (see for review (Drucker-Colin and Verdugo-Diaz 2004; Fernandez-Espejo, Armengol et al. 2005), a fraction of which has the ability to synthesize dopamine. The first clinical studies were performed in the eighties, in which autologous grafts of adrenal medulla tissue were grafted in the caudate nucleus of 2 young PD patients. Functional improvements were observed up to 10 months (Madrazo, Drucker-Colin et al. 1987), but functional recovery was mainly due to trophic effects as only 1 % of the cells synthesized dopamine. Most importantly, very few cells were detected 1 or 2 years after transplantation (Hurtig, Joyce et al. 1989). Others studies were performed using cultured chromaffin cell suspension, rendering possible cell expansion to obtain sufficient material, and sometimes differentiation prior to grafting, in order to improve the efficiency of cell grafts, including dopamine production (Drucker-Colin, Verdugo-Diaz et al. 1999). Interestingly, an *in vitro* study demonstrated the ability of chromaffin cells to form synapses with co-cultured neurons, therefore confirming the relative potential of these cells to integrate into the host (Zhang, Castell et al. 2000). Nevertheless, the major problems in all these studies were the limited level of real success (30-35 %), mainly due to the few surviving cells in long term studies, both in experimental models or in PD patients. As a consequence, this approach is no longer pursued clinically.

Finally, another type of adult cell investigated to treat PD are human retinal pigment epithelium (hRPE) cells (reviewed in (Stover and Watts 2008)). hRPE cells are supportive cells derived from the inner layer of the neural retina. For transplantation purposes, hRPE cells are isolated from human eye banks and may advantageously be expanded and stored for prolonged periods of time, while their post-mortem origins may also lead to ethical concerns. These cells exhibit TH activity but synthesise only a small amount of dopamine (Pawelek and Korner 1982). However, hRPE cells produce L-DOPA, which is synthesized as a melanin precursor, so that these cells may be used as *in situ* L-DOPA producing micro-factories in PD to supplement its oral administration. Moreover, it has been proposed that hRPE cells may be immune-privileged after transplantation due to their expression of Fas-ligand (Griffith, Brunner et al. 1995; Jorgensen, Wiencke et al. 1998).

2.2.2. Adult stem cells

Adult stem cells, with a predicted potential to better differentiate and integrate the host brain compared to the previously described adult cells, are now widely investigated for their therapeutic potential for neurodegenerative disorders. Whole bone marrow cells (i.e. HSCs & MSCs) implanted into the bilateral QA-damaged striatum of HD rat models, indicated that the transplants were viable for at least 37 days and that they reversed functional deficits such as working memory (Lescaudron, Unni et al. 2003). However, the population mostly responsible for the beneficial effects remained to be determined. More recently, transplantation of MSCs alone resulted in a decreased atrophy of rats QA-lesioned striatum (Amin, Reza et al. 2008). Interestingly, a recent study reported a functional benefit of an intravenous injection of MSCs in a QA-lesioned rat model (Edalatmanesh, Matin et al. 2009). However, in most of these studies, only a few cells (1 %) expressed neural phenotypes and it was suggested that MSCs worked as neurotrophic enhancers via the release of growth factors, therefore allowing surviving cells within the caudate to function more efficiently and to facilitate other compensatory responses (Dunbar, Sandstrom et al. 2006). In this regard, another study demonstrated the importance of factors such as stem cell factor (SCF), produced *in situ* in the lesioned striatum, to promote the migration and engraftment of MSCs via SCF receptor c-kit (Bantubungi, Blum et al. 2008). However, autologous grafts of MSCs may not be appropriate for the treatment of this genetic disease as transplanted cells would also carry the mutant huntingtin gene responsible for the disease.

In the context of PD, an interesting study reported no major differences between MSCs from normal patients compared to MSCs from parkinsonian patients, in terms of morphology, phenotype and multipotentiality, therefore confirming their potential use as autografts in this context. Moreover, in this study, up to 30 % of cells acquired dopaminergic characteristics upon differentiation *in vitro* (Zhang, Wang et al. 2008). Similarly, several teams, including ours, reported the neuronal differentiation of human MSCs toward a dopaminergic phenotype *in vitro*, indicating these cells may constitute an alternative dopamine secreting source of cells for PD (Tatard, D'Ippolito et al. 2007; Trzaska, Kuzhikandathil et al. 2007; Barzilay, Kan et al. 2008). Various animal models of PD have been developed, mainly with neurotoxins specific for dopaminergic neurons and used for evaluation of MSCs to treat PD (Li, Chen et al. 2001). Human MSCs, pre-induced towards a neuronal phenotype and transplanted in the totally dopaminergic deafferented striatum of rats, led to an improved functional recovery compared to naïve hMSC (Levy, Bahat-Stroomza et al. 2008). Another study reported a similar functional recovery with hMSCs transplanted in a

rat partial lesion model of PD, where some dopaminergic fibres are spared in the striatum (Bouchez, Sensebe et al. 2008). In this study, microdialysis demonstrated that part of the striatal pool of dopamine was restored upon MSCs transplantation. As even naïve hMSCs led to an efficient recovery of the lesion symptoms, these results may be explained by the trophic restorative effect provided by MSCs in the context of partial lesions, instead of a neuronal differentiation mechanism. Similar mechanisms of recovery were described in a study in which adipose tissue-derived MSCs secreting NGF, glial cell line-derived neurotrophic factor (GDNF) and BDNF were transplanted in the substantia nigra of rats one week after 6-OHDA lesion (McCoy, Martinez et al. 2008). Indeed, several mechanisms were proposed to explain functional recovery obtained upon human MSCs transplantation (Levy, Bahat-Stroomza et al. 2008). hMSCs, that migrate toward lesions in animal models of PD (Hellmann, Panet et al. 2006; Sadan, Bahat-Stromza et al. 2009)**,** may induce a protective or restorative effect on the remaining neurons within the host brain, due to the secretion of a large panel of neurotrophic factors, including BDNF and GDNF, which may also enhance endogenous neurogenesis (Sadan, Bahat-Stromza et al. 2009). MSCs may also modulate the host response to the lesion and probably ultimately replace functional cells within the host brain as a few MSCs with neuronal-like morphology and markers were observed in the total lesion model (Levy, Bahat-Stroomza et al. 2008). In addition, a recent study described the protection of substantia nigra dopaminergic neurons induced by hMSCs transplantation in an inflammation rat model, probably via modulation of microglia activation (Kim, Park et al. 2008). Interestingly, differentiated human MSCs may also provide a source of neurotrophin producing astrocyte-like cells. These cells, secreting BDNF, NGF and GDNF, improved the functional recovery after transplantation in the striatum of a rat model of PD (Bahat-Stroomza, Barhum et al. 2009). This growing amount of MSC therapy studies for PD may eventually set the ground for pre-clinical studies with non human primates.

Despite the difficult harvesting of NSCs from the adult brain, rendering troublesome the translation of these protocols toward the clinic, they have been evaluated for the treatment of PD and HD due to their potential to differentiate into neurons *in vivo* (Lois and Alvarez-Buylla 1993). Indeed, a study reported the transplantation of NSCs from adult rat SVZ in a QA rat model of HD, with NSC survival up to 8 weeks after grafting and migration throughout the brain. To a larger extent than MSCs, up to 15 % of adult NSCs differentiated into mature neurons with specific markers of striatal medium spiny projection neurons and interneurons (Vazey, Chen et al. 2006). To treat PD, a clinical trial has already been performed with adult NSCs (reviewed in (Arias-Carrion and Yuan 2009)), and gave

interesting proof of concept for the autologous use of these cells. NSCs were isolated from the patient's brain during the insertion of a thalamic stimulator. A total of 6 millions cells, and among them GABAergic and dopaminergic cells, were grafted 9 months later into the patient's post-commissural putamen. An improvement in the Unified Parkinson's Disease Rating Scale (UPDRS) score was observed over the next 36 months, although results returned to baseline at 5 years post-operation (Levesque 2009).

To conclude, it appears clearly that MSCs are currently the most widely investigated adult cells for brain cell therapy. However, their neuronal differentiation potential remains very low or uncertain after transplantation. In addition, the poor cell survival and engrafment observed when using chromaffin cells, hRPE cells, MSCs and in general all kinds of transplanted cells, has called into question the efficacy of such procedures. These issues may now be acknowledged by tissue engineering approaches, discussed in the following section. It is now widely admitted that cell survival, differentiation, and more generally behaviour of cells *in vivo* may be greatly enhanced using adequate biomaterial supports.

	Cells	Origins	Recipients	Scaffolds	Benefits	Reference
AD	MSC	Mouse	B-amyloid rat model		Improved cognitive abilities	Wu, Li et al. 2007
		Mouse	B-amyloid mouse model		Accelerated activation of microglia Reduction of brain amyloid beta deposits	Lee, Jin et al. 2009
HD	Sertoli		3-NP model		Possible modulation of local inflammation	Emerich 2004
	Choroid plexus	Rat	QA rat model	Alginate microcapsules	Selective neuroprotection, no evidence for sparing of striatal neurons	Borlongan, Thanos et al. 2008
	HSC+MSC	Rat	QA rat model		Reduction of working memory deficits	Lescaudron, Unni et al. 2003
	MSC	Rat	QA rat model		Decreased striatal atrophy	Amin, Reza et al. 2008
		Rat	QA rat model		Attraction of MSCs by SCF production in the striatum	Bantubungi, Blum et al. 2008
		Rat	QA rat model		IV transplantation: improved motor & cognitive performance	Edalatmanesh, Matin et al. 2009
	Adult NSC (SVZ)	Rat	QA rat model		Survival at 8 weeks, migration & 15 % of cells differentiated in mature neurons with marker of striatal medium spiny neurons	Vazey, Chen et al. 2006
PD	Adrenal chromaffin	Human	Human (autologous)		Functional improvements observed up to 10 months	Madrazo, Drucker-Colin et al. 1987
		Human	Human (autologous)		No cell detected after 1-2 years	Hurtig, Joyce et al. 1989
		Rat	6-OHDA rat model (unilateral)	Cytodex ® Glass beads	Increased survival and functional benefits (8 months)	Cherksey, Sapirstein et al. 1996
		Rat	6-OHDA rat model (unilateral)	Cytodex ® Glass beads	Increased survival and functional benefits (12 months)	Borlongan, Saporta et al. 1998
		Human	Human		Improved dopamine production with in vitro differentiation	Drucker-Colin, Verdugo-Diaz et al. 1999
	hRPE	Human	6-OHDA rat model (uni & bilateral)	Gelatine microcarriers (Spheramine ®)	Functional recovery with chronic inflammation at late time-point	Cepeda, Flores et al. 2007
		Human	MPTP primate model	Gelatine microcarriers (Spheramine ®)	Long term functional improvements an cell survival (18 months) No immunosuppression required	Doudet, Cornfeldt et al. 2004
		Human	Human	Gelatine microcarriers (Spheramine ®)	Open-label study: good tolerability, sustained motor clinical improvement up to 6 months after grafting Currently: phase II double-blind, randomized, multicenter, placebo controlled clinical	Bakay, Raiser et al. 2004
	MSC	Mouse	MPTP mouse model		Functional recovery, survival at 35 days and TH expression	Li, Chen et al. 2001
		Rat	6-OHDA rat model (unilateral & partial)		Adipose tissue-derived MSCs secreting BDNF, GDNF & NGF Functional recovery after transplantation in SN but no neuronal differentiation *in vivo*	McCoy, Martinez et al. 2008
		Human	6-OHDA rat model (unilateral & partial)		Induction of MSCs to secrete GDNF & BDNF Transplanted on the day of lesion, MSCs migrated toward the lesioned striatum and had a regenerative effect	Sadan, Bahat-Stromza et al. 2009
		Human	6-OHDA rat model (unilateral & total)		Improved functional recovery with differentiated cells (TH expression and DOPA secretion)	Levy, Bahat-Stroomza et al. 2008
		Human	6-OHDA rat model (unilateral & partial)		Trophic, restorative effect of MSCs	Bouchez, Sensebe et al. 2008
		Human	6-OHDA rat model (unilateral & partial)	PAMs with LM surface & releasing NT3	Functional recovery with combination of PAMs and MIAMI cells	Delcroix, Garbayo et al. Manuscript under preparation
	Adult NSC	Human	Human (autologous)		Improved UPDRS score over 36 months Back to baseline 5 years post-operation	Levesque 2009

Table 2. Adult cell therapies for neurodegenerative disorders

Abbreviations: AD: Alzheimer's disease; BDNF: brain-derived neurotrophic factor; GDNF: glial cell line derived neurotrophic factor; HD: Huntington's disease; hRPE: human retinal pigment epithelium; HSC: hematopoietic stem cell; IV: intravenous; LM: laminin; MIAMI: marrow isolated adult multilineage inducible; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSC: mesenchymal stem cell; NP: nitropropionic acid; NSC: neural stem cell; NT3: neurotrophin 3; OHDA: hydroxydopamine; PAMs: pharmacollogically active microcarriers; PD: Parkinson's Disease; QA: quinolinic acid; SCF: stem cell factor; SN: substantia nigra; UPDRS: unified Parkinson's disease rating scale.

3. ADULT CELL THERAPY FOR BRAIN NEURONAL DAMAGES: IMPROVEMENTS WITH TISSUE ENGINEERING

Tissue engineering combines cells with a supportive element called a scaffold, with the ultimate goal of repairing organs and tissues. A scaffold may be of various compositions and shapes, and may improve cell behaviour, due to the 3D environment as well as the mechanical and signalling cues they provide to transplanted cells. Indeed, scaffolds may stimulate the cells to express for example neuronal molecules in the context of a neuronal disorder. Tissue engineering 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 foetal NSCs, owing to their natural ability to integrate and differentiate within the brain. Benefits gained using these cells in combination with a tissue engineering approach now have to be translated to adult cells such as MSCs, in order to improve their survival *in vivo* as well as to guide their differentiation and integration within the host brain.

In the following section, we describe the various types of scaffolds and their required properties for implementation in tissue engineering applications in the brain. We will then review the past and current applications of brain tissue engineering in combination with adult cells. Thereafter, the improvements that may be achieved with modifications of classical scaffolds by means of a biomimetic approach, associating extracellular matrix molecules (ECM) components, will be described. Where necessary, preliminary studies made on model cells which led to the current knowledge now applied to adult cell engineering will be described. We will finish by describing a novel approach currently developed in our laboratory, the pharmacologically active microcarriers.

3.1. Scaffold properties for brain cell therapy

Scaffolds have been primarily developed in the context of connective tissue disorders for adult cell therapy, mainly with MSCs, due to their osteogenic (Kim, Kim et al. 2003) and chondrogenic (Richardson, Curran et al. 2006) differentiation potentials. Therefore, implementation of tissue engineering in combination with adult cells in the particular context of brain therapy still remains an emerging field with many limitations. Indeed, if a small size is crucial for implantation into the brain, scaffolds must also be fully biodegradable and biocompatible for surgical use, minimizing macrophagic and microglial reaction, without inducing neurotoxicity (see for review (Menei, Montero-Menei et al. 2005)). In this section,

we will describe the major parameters that must be taken into account for brain tissue engineering.

The first consideration in scaffold tailoring for brain is size. Scaffolds should necessarily be small enough to be easily implanted into the skull cavity, either via stereotactic implantation or under neuronavigation, therefore allowing an implantation in discrete and precise areas of the brain. Moreover, small-sized scaffolds render repeated implantations possible, with no need for open-surgery (Menei, Montero-Menei et al. 2005). In this sense, microstructured and nanostructured scaffolds, produced by various techniques (reviewed in (Seidlits, Lee et al. 2008) may be used.

Scaffolds are based either on natural or on synthetic biomaterials used alone or in mixtures, providing scaffolds with different properties (see for review (Potter, Kalil et al. 2008; Dalton and Mey 2009)). Scaffolds based on biodegradable gels encapsulating various molecules and cells have been widely studied, with e.g. polyethylene glycol (PEG) (Namba, Cole et al. 2009), diblock copolypeptide (Yang, Song et al. 2009) or hyaluronic acid (Wang and Spector 2009) hydrogels. However, gel-based scaffold strategies most of the time require open-surgery, unless an *in situ* gelling process is used. For example, a carboxymethylcellulose (CMC), poly(ethyleneimine) and rat MSCs mixture injected subcutaneously forms a solid gel containing MSCs at physiologic temperature (Kim 2009). Similarly, diblock copolypeptide gel-based scaffolds may also have the properties to become solid after injection into the brain (Yang, Song et al. 2009). Particulate scaffolds, that may be constituted of aliphatic polyesters, including poly(lactic-co-glycolic acid) (PLGA), overcome this problem and have been intensively studied with many cell types, and among them embryonic cells and NSCs (Newman and McBurney 2004; Bible, Chau et al. 2009). Depending on scaffold shapes, cell-material interactions may be advantageously increased due to a large specific surface as it is the case for microsphere-shaped scaffolds. Scaffolds based on nanofibrous technology may also allow a higher surface area and a high porosity required for cell adhesion (Cao, Liu et al. 2009), with more original approaches such as nanofibrous spiral scaffolds aiming at further improving cell attachment (Valmikinathan, Tian et al. 2008).

Others parameters of tremendous importance are the biocompatibility and biodegradability of the biomaterials used for scaffold preparation (Vert 2009; Yang, Song et al. 2009). Indeed, the adverse host cell response, such as glial scar and inflammation, after scaffold implantation have to be minimized (see for review (Fournier, Passirani et al. 2003). For example, implantation of PLGA microspheres into the brain does not induce a specific astrocytic reaction. A similar overexpression of glial fibrillary acid protein (GFAP) was

observed within the first 2 weeks after microsphere transplantation or the injection of media alone, before persisting as a scar along the injection tract. At the same time, macrophage and microglia were observed early after implantation, but disappeared while microspheres remained within the tissue with no T-lymphocyte reaction detected (Menei, Montero-Menei et al. 2005). It is interesting to note that size of particles may also affect the extent of the host response. *In vitro*, small size phagocytable hydroxyapatite particles (1-30 µm) have been shown to induce a strong production of the inflammatory cytokines TNFα, IL6 and IL10 by human monocytes, the first cells recruited to the inflammation site, which may be correlated to a stronger host inflammatory response. On the other hand, this effect decreased for particles of more than 30 µm in diameter. Importantly, shape is also critical for the extent of the response, needle-shape being potentially more detrimental compared to spherical-shaped particles (Laquerriere, Grandjean-Laquerriere et al. 2003). Similarly, no phagocytosis was observed 2 months after transplantation of PLGA microspheres of 30 µm in diameter within rat striatum (Veziers, Lesourd et al. 2001). In addition, scaffolds should be able to degrade with time, with degradation products that may also be eliminated by the host, allowing a full integration of transplanted cells into the brain. Again, PLGA is known to be fully degradable, with end-product metabolites being CO_2 and H_2O (Menei, Montero-Menei et al. 2005). Importantly, the degradation products must also be biocompatible, a criteria not observed for synthetic poly(methylidene malonate 2.1.2) microspheres implanted into rat brains even if biocompatibility of the intact microspheres was satisfactory (Fournier, Passirani et al. 2006).

Surface characteristics, such as charge, hydrophilicity and hydrophobicity, are also of great importance for cell therapy purposes. Indeed, transplanted cell attachment critically depends on the polymer surface charges, cells being attracted to positive charged surface due to sialic acid residues on the cell membrane which produce a net negative charge on the cell surface. Furthermore, the first step following implantation of a scaffold within the brain is its coverage by a non-specific layer of proteins, a process mainly governed by surface hydrophobicity and hydrophilicity, which may contribute to the inflammation process and biocompatibility problems (Fournier, Passirani et al. 2003).

Topography, i.e. outer architecture, but also inner architecture of scaffolds also affects their behaviour once implanted into the brain. For example, the presence of pores and channels on the surface of synthetic poly-ε-caprolactone scaffolds may enhance host astrocytic infiltration and affect host cell migration (Wong, Krebsbach et al. 2008). Moreover, access to nutrients is a critical parameter for neuronal cells, which requires large amounts of nutrients such as glucose. Therefore, if larger implants are used, vascularisation is required for

cell survival. In this sense, porous scaffolds, or scaffolds that become porous after implantation during degradation, may alleviate vascularisation problems. Surface topography may also affect the behaviour of the transported cells. Recently, Anselme et al. described the effects of titanium and stainless steel surfaces with various amplitudes of roughness on human MSC adhesion and spreading in culture. This study demonstrated that cells were disturbed when the distance between peaks on the surface was similar to cell size (Anselme 2009). Similarly, another study demonstrated an enhanced differentiation of human MSCs in contact with micropatterned surfaces (Wang, Itaka et al. 2009). To exemplify the growing importance of this field, an emerging concept named Materiomics is now under development for the high throughput screening of material topography effects on cells (Blitterswijk, Stamatialis et al.).

Finally, a very interesting study recently described the effects of matrix elasticity to direct MSCs lineage specification, with soft type matrices, therefore mimicking brain, being neurogenic while stiffer matrices appeared to be myogenic and furthermore osteogenic. The observed phenotypic specification was irreversible after several weeks in culture, therefore reflecting neuronal commitment of the MSCs cultivated on soft matrices. Again, this study underlined the importance of the choice of the biomaterial for brain tissue engineering (Engler, Sen et al. 2006).

3.2. Applications for adult cell therapy of the damaged brain

Adult cells may advantageously be used for therapeutic purposes in the brain in combination with tissue engineering strategies (table 1, 2 and figure 4). The following section reviews primary studies that investigated the potential of dextran and alginate-based scaffolds in combination with adrenal chromaffin cells and choroid plexus cells to treat PD and HD, respectively. Studies on hRPE cells in combination with gelatine-based scaffolds, a device currently under clinical trials (Phase IIb) for the treatment of PD will also be described, as well as collagen scaffolds seeded with hMSCs for the treatment of TBI. These scaffolds mainly provide an adequate anchorage to the transported cells. Collagen has also been primarily widely used for cell therapy purposes in the brain due to its high cell adhesion properties (Heckmann, Fiedler et al. 2006). Nevertheless, one study reported that 3D PLGA sponges, containing collagen and adrenal chromaffin cells, upregulated cell viability and metabolic function in culture (Elcin, Elcin et al. 2003).

3.2.1. Particulate scaffolds

Proof of concept for the use of particulate scaffolds has been obtained in the nineties with adrenal chromaffin cell grafts in animal models of PD. As previously described in section 2.2.1, the major problems encountered at that time with chromaffin cells was the poor cell survival and the absence of long term effects *in vivo* (Drucker-Colin and Verdugo-Diaz 2004). Two studies demonstrated an enhanced survival of adhered cells onto collagen-coated dextran (Cytodex 3 ®) or glass bead microcarriers into the brain of hemi-parkinsonian rats, with functional benefits after 8 months (Cherksey, Sapirstein et al. 1996) and 12 months post-transplantation (Borlongan, Saporta et al. 1998). Both types of beads resulted in similar improvements of cell survival, but the mechanisms were not studied. Moreover, it is noteworthy that no inflammation was detected when implanted into the striatum. The pivotal finding of these studies is that adult rat adrenal chromaffin cells implanted in the brain after attachment to microcarriers retain their ability for prolonged times (12 months) to correct a striatal dopamine deficit as judged by their efficacy in reducing apomorphine-induced rotation. During the same period, these scaffolds were used for transplanting human FVM cells in similar rat model of PD (Saporta, Borlongan et al. 1997). Interestingly, the use of Cytodex ® microcarriers resulted in an increased cell survival, without immunosuppression. This effect was thought to result from the presence of a protective astrocytic cloak around the cell/microcarrier complexes. As glass and Cytodex ® microcarriers gave similar results, these data demonstrated, for the first time, the need for cell attachment to a 3D complex to improve the survival of transplanted cells. Moreover, it also underlined the immunomodulatory benefits that may be gained from the use of a tissue engineering strategy.

hRPE cells, described in section 2.2.1, have been used in combination with gelatine microcarriers for the treatment of PD under the name Spheramine ® (for review, see (Stover and Watts 2008)). Spheramine ® associates cultured hRPE cells attached to biocompatible, but non biodegradable cross-linked porcine gelatine microcarriers, with a mean diameter of 100 µm. All components are produced under current good manufacturing practices. Several studies in Parkinsonian rat models (unilateral and bilateral lesions) have proven the efficacy of Spheramine ® (Watts, Raiser et al. 2003), with an increased survival of hRPE cells, without immunosuppression, and long term functional improvements. However, chronic inflammation was observed at later time-points (5 months) (Flores, Cepeda et al. 2007). These microcarriers were also implanted in the brain of hemi-parkinsonian monkeys and resulted in long term cell survival and functional improvements at 18 months (Doudet, Cornfeldt et al. 2004). Importantly, hRPE cells unattached to microcarriers do not well survive in the brain,

and do not produce a lasting therapeutic effect in various PD animal models. These encouraging results led to an open-label clinical study that included 6 patients with advanced PD receiving 325000 hRPE cells attached to microcarriers and demonstrated a good tolerability to Spheramine®. Moreover, at 6 months post-operation, the mean UPDRS-M (off) score improved to 34 % from the pre-operation baseline. Half of the patients also demonstrated a reduced Dyskinesia Rating Scale scores (Bakay, Raiser et al. 2004). The success of this strategy finally led to conduct a phase II double-blind, randomized, multicenter, placebo-controlled (sham surgery) study to evaluate safety, tolerability, and efficacy of Spheramine ® implanted bilaterally into the postcommissural putamen of patients with advanced PD (Stover and Watts 2008). Being already under phase II clinical trials, the concept of Spheramine ® is currently the most advanced tool that successfully combines adult cell therapy and tissue engineering for treatment of PD. Nevertheless, the mechanisms underlying the improved survival of hRPE cells upon attachment to gelatin are not yet well elucidated (Stover and Watts 2008).

We can finally mention an interesting study in the context of HD, in which transplants of encapsulated adult rat choroid plexus cells were used due to their growth factor production potential of basic fibroblast growth factor (bFGF), BDNF, GDNF, hepatocyte growth factor (HGF), IGF, NGF and neurotrophins (NT3-4) (Borlongan, Thanos et al. 2008). Again, the idea underlining cell encapsulation in alginate beads was to improve cell viability and to prevent host rejection. Transplantation of these cells into rat striatum resulted in an encouraging neuroprotection when a QA lesion was performed 3 days after cell transplantation, even if no evidence was provided concerning the sparing of GABAergic medium spiny projection neurons, especially sensitive to degeneration in the context of HD.

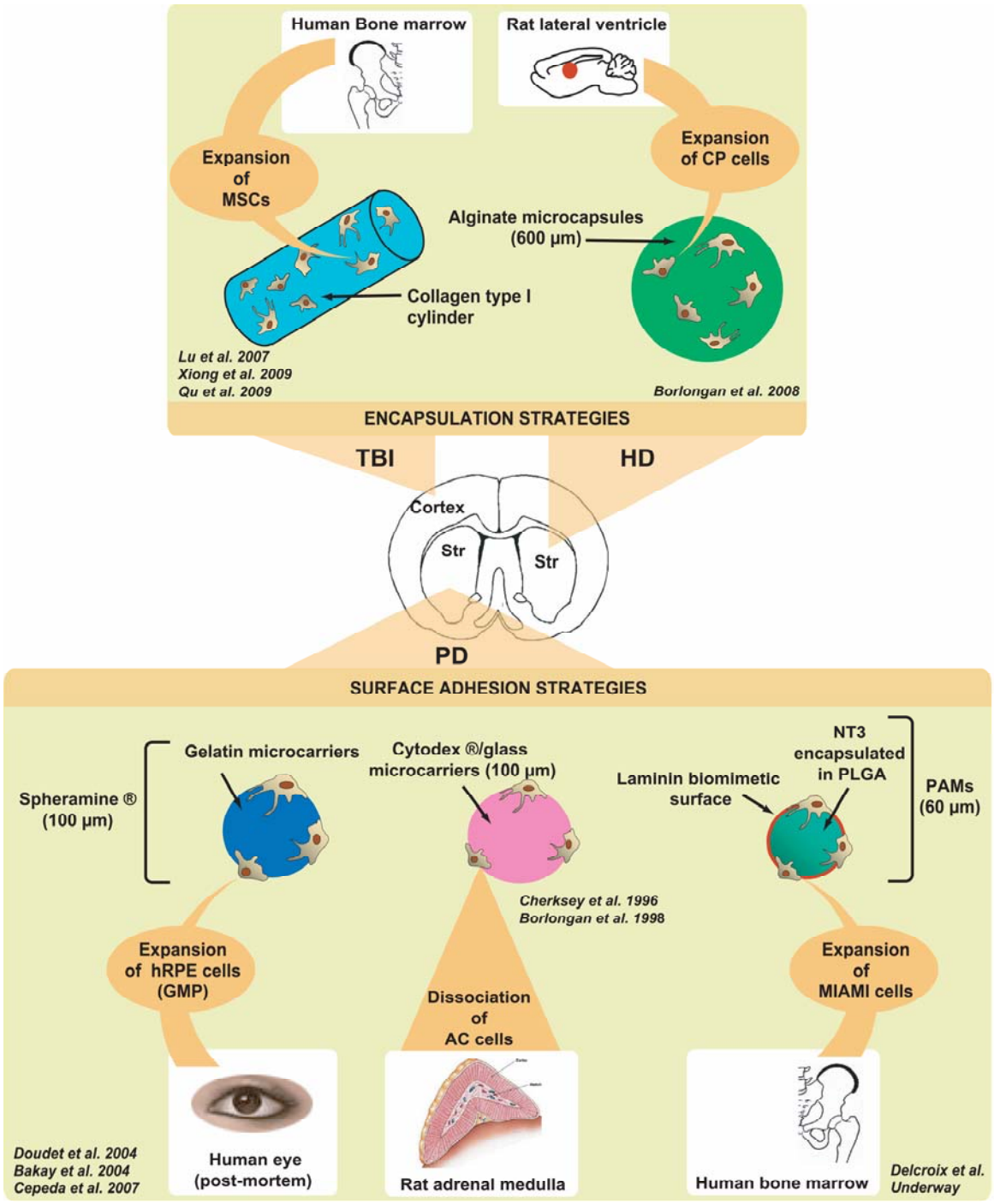

Figure 4. Adult cells for tissue engineering to repair neuronal-damaged brain

Several types of scaffolds have been used to repair or protect damaged brains, either in animal models or in human as it is the case for Spheramine ®. Scaffolds may be classified in 2 families, those that encapsulate cells and the ones that transport cells on their surface. Noteworthy, the concept of PAMs transporting MIAMI cells is a unique approach combining adult stem cells and tissue engineering to treat PD. Abbreviations: AC: adrenal chromaffin, CP: choroid plexus, HD: Huntington's disease, hRPE: human retinal pigment epithelium, MIAMI: marrow isolated adult multilineage inducible, MSCs: mesenchymal stem cells, NT3: neurotrophin 3, PAMs: pharmacologically active microcarriers, PD: Parkinson's disease, PLGA: poly(lactic-co-glycolic acid), Str: striatum, TBI: traumatic brain injury.

3.2.2. Gel-based scaffolds

Use of gel-based scaffolds, without cells, may be advantageous to repair the brain after an ischemic stroke. For example, Yamashita et al. described the development of a porous gelatine-siloxane hybrid that contributed to maintain brain's integrity, together with an integration of host cells inside the marginal cavities of the scaffold (Yamashita, Deguchi et al. 2009). In the context of TBI, collagen scaffolds did not reduce the lesion size nor did they improve functional recovery, unless if seeded with hMSCs (Lu, Mahmood et al. 2007). Indeed, four days after TBI, transplantation of a cylindrical collagen scaffold seeded with $3x10^6$ hMSCs in the lesion cavity induced a reduction in the lesion volume, together with an improved spatial learning and sensorimotor function. The scaffold was readily degradable and was already not detectable 35 days after TBI. At that time, a decreased tissue loss from cortex was also observed, together with an enhanced migration of MSCs within the parenchyma. Recent studies performed by the same team revealed that delayed transplantation of these complexes (7 days after TBI instead of 4 days) further enhanced their benefits, in terms of spatial learning and sensorimotor function, angiogenesis in the injured cortex as well as transcallosal fibre length (Xiong, Qu et al. 2009). The same team confirmed these results in a mouse model of TBI (Qu, Xiong et al. 2009). In experimental models of TBI, MSCs are usually injected adjacent to the lesion within the parenchyma to avoid injection within the lesion cavity. An advantage of this strategy was the possible use of 3 fold more MSCs compared to parenchymal injection, therefore increasing the regenerative potential. However, the major limitation was the need for open-surgery to implant the device. This issue may potentially be addressed using an *in situ* gelling process, previously described in section 3.1, even if the possible expansion and ensuing damage to the brain parenchyma of the solidifying gel has to be taken in consideration. Nevertheless, all these studies demonstrate the need for a 3D support to improve cell survival, even if the underlying mechanisms are not always well-understood.

3.3. Scaffold modifications-Biomimetic approach

To further and better regulate cell behaviour, several strategies are now focusing not only on improving cell interactions with the biomaterials by modifying the scaffold surface with collagen, but also on studying and implementing the different signals that biological molecules, mainly derived from the ECM, will provide the cells when presented within a scaffold. We will first describe cell-ECM molecule interactions and the resulting cell behaviour, as well as some studies evaluating the benefits of such "biomimetic scaffolds",

mainly *in vitro*. Finally, the pharmacologically active microcarriers (PAMs) developed in our laboratory, that constitute a more advanced approach combining a bioactive surface with the controlled delivery of a growth factor will be presented.

3.3.1. Cell-ECM interactions

The ECM is composed of various molecules that interact with cells *in vivo* and regulate numerous processes during organism development as well as throughout life; this being the rationale for their use in tissue engineering. Briefly, the ECM is composed of 2 major components, the basement membrane and the interstitial matrix, containing adherent glycoproteins, glycosaminoglycans and ions. ECM proteins, such as collagen, fibronectin (FN), laminin (LM), tenascin and proteoglycans, interact with each other forming a tissue scaffold (see for review (Bosman and Stamenkovic 2003)). ECM is continuously remodelled via ECM molecule production and degradation. Composition and mostly proportion of its constituents vary depending on the type of tissue giving different mechanical, chemical or signalling cues to the surrounding cells. In the present review, we have chosen to focus on FN and LM, widely studied for brain cell therapy due to their astonishing variety of effects on cells, including adhesion, proliferation, migration and differentiation.

FN and LM structures have previously been described (see (Hynes and Yamada 1982; Powell and Kleinman 1997)). FN is a "V" shaped dimer of 2 almost identical linear polypeptides of 235 kD linked by a disulfide bond at the C-terminal end. Monomers are constituted of 3 domains (FN-1, FN-2 and FN-3), each having specific interaction sites with other ECM molecules or cell surfaces. LMs are large (400 to 900 kDa) crucifix or "T"-shaped mosaic molecules with two or three short arms and one long arm. Similarly to FN, arms of LM are composed of many distinct domains with different structures and functions. Sites for receptor-mediated cell attachment and promotion of neurite outgrowth reside in the terminal region of the longest arm (Engvall and Wewer 1996). At least 18 types of LMs have been described (Durbeej 2009), being mostly formed by 3 chains, plus some variants. Five long α chains, 3 β chains and 3 γ chains have been reported. The new nomenclature for LM now refers to chain numbers, e.g. LM 1, constituted of chains α1, β1 and γ1, should now be termed LM 111 (Aumailley, Bruckner-Tuderman et al. 2005).

LM and FN interact with cells via the integrin family of receptors, therefore allowing cell attachment to the matrix and further signal transduction. Integrins are a family of proteins constituted of an α subunit and a β subunit. 8 β and 18 α subunits have been described, which assemble into 24 distinct integrin receptors, all having specific binding affinities with ECM

molecules such as FN and LM (Barczyk, Carracedo et al. 2009). The interactions between ECM molecules and integrins have been fully reviewed, as well as the highly complex downstream signalling pathways that originate at the focal adhesion sites, where a variety of proteins (e.g. Ilk, Fak, Src) interact with the integrin tails on their cytoplasmic ends. This interaction regulates cell behaviour such as survival, proliferation, migration and differentiation (Hynes 2002). As mentioned above, integrins bind with LM and FN via specific domains of these proteins. For example, both these proteins possess a RGD peptide fragment, known to interact with about one third of the integrins (Ruoslahti 2003), but, to our knowledge, without inducing a downstream signal on its own. In addition to RGD peptides, integrins may bind additional sites present on ECM proteins, guiding specific downstream events (Pollard and Earnshaw 2004). Others fragments of LM, mainly from the E8 domain, e.g. the peptides IKVAV & YIGSR have been isolated and play crucial signalling roles mainly in neurite outgrowth and cell adhesion, respectively (Powell and Kleinman 1997; Orive, Anitua et al. 2009). Accordingly, numerous studies aim at functionalizing scaffolds with similar peptide fragments to reproduce the effect of the native ECM molecules, but also to isolate one of its specific effects.

3.3.2. ECM molecules and scaffolds: control of cell behaviour

Potential roles of ECM molecules for brain repair, collagen due to its high cell adhesion properties, but more especially FN and LM due to their cell signalling potentials, are now widely admitted. *In vivo*, endogenous levels of FN and LM increase in TBI context, suggesting a reparative role of these molecules (Tate, Tate et al. 2007). Moreover, *in vivo* reports describe the role of LM in axonal regeneration of the central nervous system (Grimpe, Dong et al. 2002). Tissue repair benefits have also been obtained by transplanting LM-based hyaluronic gel scaffolds, without cells, in a rat model of cortical lesion (Hou, Xu et al. 2005). As aforementioned, the very diverse effects of these ECM molecules, which include cell proliferation, survival as well as differentiation, were primarily demonstrated for brain cell therapy using PC12 cells, cultured DRG neurons and foetal neural stem cells. An interesting study describes the improved integration as well as tissue regeneration obtained with transplantation of a mixture of mouse embryonic cells and of LM (Kearns, Scheffler et al. 2006). In this *ex-vivo* brain slice culture model of Parkinson's disease, LM also accelerated the migratory potential and process extension of transplanted cells. Cell migration may also be affected by the presence of LM and FN. These effects were also observed in different models using foetal NSCs asscociated to LM and/or FN both *in vitro* (Kearns, Laywell et al. 2003; Tate, Garcia et al. 2004) and *in vivo* within a scaffold (Tate, Shear et al. 2002).

Nowadays, an increasing amount of studies investigate the effects of ECM molecules on cultured adult cells, mainly MSCs, in order to implement the use of biomimetic scaffolds for adult cell therapy (table 3).

Effects	ECM component or derived peptides	Observations	Reference
Survival	*RGD*	PEG hydrogel RGD improved hMSCs survival *in vitro* Improvement enhanced with the use of a glycine spacer between RGD and the gel	Salinas and Anseth 2008
	RGDSP & IKVAV	PEG-hydrogel Both peptides improved hMSCs survival Combination of IKVAV with degradable gels decreased the benefits compared to non-degradable gels	Jongpaiboonkit, King et al. 2008
	FN & fibrinogen	Single-cell encapsulation of hMSCs in FN- or fibrinogen-containing hydrogel capsules Enhanced survival of cells + increased cell metabolic activity *in vitro* Validation of the concept *in vivo* (uninjured hindlimb model in rats)	Karoubi, Ormiston et al. 2009
Proliferation	*Collagen I*	PLCL/collagen I nanofibrous scaffolds Increased proliferation of hMSCs *vs* standard PLCL scaffolds Effect due to adequate cell adhesion properties of collagen I Allow neuronal differentiation of hMSCs when exposed to the appropriate inducers	Prabhakaran, Venugopal et al. 2009
	FN	FN substrate Increased proliferation of MIAMI cells *in vitro*	Delcroix, Garbayo et al. Manuscript under preparation
Neuronal differentiation	*LM*	LM 111 substrate Induction of nestin expression by MSCs *in vitro*	Ho, Yu et al. 2006
		LM substrate Morphological changes in hMSCs during neuronal differentiation *in vitro* Increased percentage of cells with secondary and tertiary branching on LM *vs* FN or PDL	Qian and Saltzman 2004
		LM substrate Increased cell length and expression of neuronal proteins (3-Tubulin and NFM) during differentiation of MIAMI cells *in vitro vs* FN or glass	Delcroix, Garbayo et al. Manuscript under preparation

Table 3. Control of MSCs behaviour by ECM components

Abbreviations: ECM: extracellular matrix molecules; FN: fibronectin; LM: laminin; MIAMI: marrow isolated adult multilineage inducible; MSC: mesenchymal stem cell; NFM: neurofilament medium; PDL: poly-D-lysine; PEG: polyethyleneglycol; PLCL: poly(L-lactic acid)-co-poly-(ε-caprolactone).

Survival. The anti-apoptotic role of ECM molecules such as FN and LM has been demonstrated *in vitro* with foetal NSCs and neuronal cells (Gibson, Craig et al. 2005; Hall, Lathia et al. 2008). Using these types of cells and collagen or PLGA scaffolds modified with LM or FN, cell survival and functional improvements were observed in animal models of TBI and stroke (Bible, Chau et al. 2009; Tate, Shear et al. 2009). Similarly, a recent study demonstrated an enhanced survival *in vitro* of human MSCs within a PEG hydrogel modified with RGD peptides. In this study, RGD peptides attached to the scaffold provided the required adhesion sites to maintain MSC survival, while soluble peptides resulted in a strong decrease of cell viability. Noteworthy, the presence of a glycine spacer between the RGD peptides and the gel improved further MSC survival, therefore underlining the importance of the presentation context of the peptide within the gel (Salinas and Anseth 2008). Another team described the effect of FN & LM-derived peptides (RGDSP and IKVAV, respectively) using

a PEG-hydrogel array to screen the effect of these ECM molecules on hMSCs viability. Their strategy allowed to also analyse the consequences of the hydrogel used, for example, the combination of the IKVAV peptides with degradable gels resulted in decreased sustained viability compared to non-degradable gels (Jongpaiboonkit, King et al. 2008). Using a subpopulation of hMSCs, preliminary results of our team show in a rat model of global ischemia, that marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito, Diabira et al. 2004) adhered onto PLGA fibronectin-coated microspheres enhance the neuroprotection effect observed with the cells alone (Garbayo, Curtis et al. 2009, manuscript under preparation). This effect may be attributed to an increased survival of the cells on these carriers, or the increased production of neuroprotective mediators by these cells or both. A recent study described the single-cell encapsulation of human MSCs in FN- and fibrinogen-containing hydrogel capsules in order to rescue the cells from anoikis, a loss of anchorage-induced apoptosis. One of the advantages of this strategy was to ensure the metabolic demand of encapsulated cells compared to larger scaffolds that may limit nutrient access. Therefore, the immobilized matrix molecules within the gel resulted in an enhanced survival of cells in culture as well as in an increased cell metabolic activity, effects that were certainly mediated via the MAPK/ERK signalling cascade and upstream integrin/ECM molecule interactions. Interestingly, upon cell encapsulation, the authors observed an increased cell retention in uninjured hindlimb of rats (Karoubi, Ormiston et al. 2009).

Proliferation. It is now well-accepted that ECM molecules may affect proliferation as well as life span of cells, this being the rationale for expanding MSCs on ECM molecules in several *in vitro* protocols (see for example (D'Ippolito, Diabira et al. 2004; Matsubara, Tsutsumi et al. 2004). Cell proliferation is not only controlled by soluble mitogens but also by components of the ECM. For example, modification of PLCL (poly(L-lactic acid)-co-poly-(ε-caprolactone)) nanofibrous scaffolds with collagen I resulted in an increased proliferation of human MSCs compared to standard PLCL scaffolds (Prabhakaran, Venugopal et al. 2009). Moreover, in this study, neuronal-like hMSCs were observed on the surface of the PLCL/ColI scaffolds after exposure to the adequate induction media. The benefits of collagen I was assumed to result from its high cell adhesion properties, thought to be required to ensure a proper neuronal differentiation. In our laboratory, we observed an increased proliferation of the human stromal MIAMI cells expanded on FN compared to standard culture substrates (Delcroix, Garbayo et al. manuscript under preparation). Extensive crosstalk takes place between integrin and growth factor receptor signalling pathways to stimulate progression through the G1 phase of the cell cycle, via induction of G1 cyclins and suppression of

inhibitors of the G1 cyclin-dependent kinases. Indeed, mitogenic signalling may be weak and transient in the absence of integrin-mediated cell adhesion for cells with an anchorage dependent growth, such as MSCs (see (Danen and Yamada 2001) for more details on the involved mechanisms).

Differentiation. Neural precursor differentiation is certainly one of the first and most studied effects of ECM molecules, especially with LM as well as its derived bioactive peptides. Indeed, LM, and especially LM 211 (Bosman and Stamenkovic 2003), was first described to enhance neurite outgrowth of neurons in the early eightees (Rogers, Letourneau et al. 1983) There are at present many studies reporting an effect of LM on neurite length as well as cell proliferation (Hammarback, Palm et al. 1985; Rogers, Palm et al. 1988; Freire, Gomes et al. 2002; Rankin, Guy et al. 2008). LM-derived peptides of various lengths, containing YIGSR, and IKVAV sequences, have been isolated and are responsible for neurites outgrowth of DRG neurons (Shaw and Shoichet 2003). As a result of these studies, many experiments assessed the potential of various biomimetic scaffolds to induce these effects in cultured DRG neurons and PC12 cells *in vitro*, such as micropatterned PLGA particles modified with the LM-derived peptide PPFLMLLKGSTR (Yao, Wang et al. 2008), PLGA LM/collagen 1 hydrogels (Deister, Aljabari et al. 2007) or LM-modified electrospun scaffolds (Koh, Yong et al. 2008).

Most uses of ECM molecules for adult cell tissue engineering aimed at bone reconstruction by promoting MSCs osteo- and chondrogenic differentiation (Docheva, Popov et al. 2007). As examples, collagen adsorbed to PLGA is known to promote osteogenic differentiation of hMSCs whereas a PCL-vitronectin substrate does not (Deister, Aljabari et al. 2007). LM type 332 may be a major inducer of MSCs toward the osteogenic lineage (Klees, Salasznyk et al. 2007). Nevertheless, in the context of neuronal differentiation on which we focus in this review, LM may also have a major role. For example, a team compared different surface chemistries and demonstrated that a substrate of LM 111 alone induced nestin expression by MSCs, a marker specific of neural precursors, (Ho, Yu et al. 2006). This result suggests a bioactive signalling role for LM 111 in the induction of MSCs toward a neuronal lineage. There are now evidences that LM may also induce morphological changes in MSCs during neuronal differentiation *in vitro* (Qian and Saltzman 2004), as primarily observed in PC12 cells. In this study, the authors described an increased percentage of human MSCs exhibiting secondary and tertiary branching during differentiation on a LM substrate compared to FN or PDL. In our laboratory, we also observed an increased cell length and expression of neuronal proteins (β3-Tubulin and NFM) accompanied by a decreased proliferation when the human mesenchymal stromal MIAMI cells were differentiated on LM

compared to FN or glass *in vitro* (Delcroix, Garbayo et al., manuscript under preparation). As a result of these studies, we are now investigating a tissue engineering strategy using the pharmacologically active microcarriers (PAMs) with a biomimetic LM surface to favour MSCs neuronal differentiation in the context of PD.

Interestingly, blend of polymers are now investigated to combine the benefits of individual components in order to design scaffolds with particular mechanical and bioactive properties (Garcia-Fuentes, Meinel et al. 2009; Prabhakaran, Venugopal et al. 2009). However, despite several promising proof of concepts, intensive efforts still have to be made to unravel all the possibilities offered by combining tissue engineering and adult cell therapy to better control cell fate *in vivo*.

3.3.3. The pharmacologically active microcarriers: a tool to combine the biomimetic approach & the controlled release of a growth factor

In addition to the biomimetic approach, another way to improve the efficiency of cell grafts is to deliver a growth factor by the transplanted scaffolds, further affecting the fate of both transplanted and host cells (see for review (Tatard, Menei et al. 2005)). Our group has managed to encapsulate therapeutic proteins within PLGA microspheres which deliver them in a sustained and controlled manner. The use of these growth factor delivery vectors for neuroprotection or for the repair of the nigro-straital dopaminergic system has been successfully validated in animal models of AD or of PD, respectively (Pean, Menei et al. 2000; Jollivet, Aubert-Pouessel et al. 2004; Menei, Montero-Menei et al. 2005). In this sense, we developed the pharmacologically active microcarriers (PAMs) that combine these two approaches. These PAMs are biodegradable and biocompatible PLGA microspheres conveying cells on their surface, therefore providing an adequate 3D microenvironment *in vivo*. Moreover, the controlled delivery of a trophic factor in combination with a biomimetic surface act synergistically to stimulate the survival and/or differentiation of the grafted cells toward a specific phenotype, therefore enhancing their engraftment after their complete degradation (Tatard, Venier-Julienne et al. 2005). Finally, it should be noted that the delivered molecule may also affect the host microenvironment allowing the integration of the grafted cells and/or stimulating the lesioned brain repair capacities (figure 5).

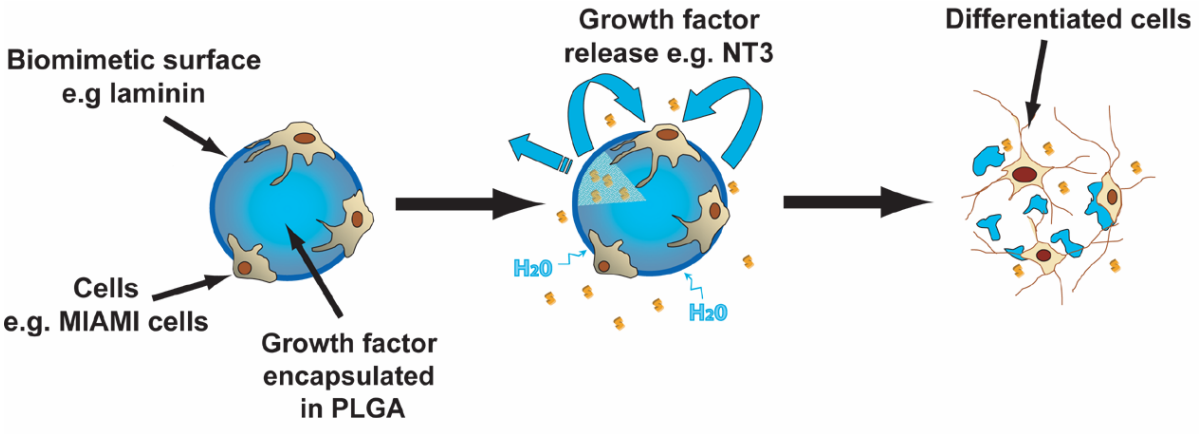

Figure 5. PAMs in combination with adult mesenchymal stromal MIAMI cells for brain cell therapy

PLGA microspheres coated with laminin and releasing NT3 in a controlled fashion are used as cell carriers which can then be grafted. NT3 and laminin should work together to influence the survival and differentiation of MIAMI cells as well as the microenvironment. After complete degradation of the microparticles the cells can integrate the parenchyma. Abbreviations: PAMs: pharmacologically active microcarriers, PLGA: poly(lactic-co-glycolic acid), NT3: neurotrophin 3, MIAMI: marrow isolated adult multilineage inducible. Adapted with permission from Tatard et al., Biomaterials, 2005.

The efficacy of this unique and simple device of cell and protein-delivery in neuroprotection and tissue repair for the treatment of neurodegenerative diseases has been validated in a PD rat model. We have first shown that their small size allows their stereotactic implantation in precise and functional areas of the brain without causing damage to the surrounding tissue. The proof of concept was obtained with a neuronal cell line (PC12 cells) transported by NGF-releasing PAMs (Tatard, Venier-Julienne et al. 2004) (figure 6). The efficacy of PAMs for cell therapy of PD in a clinical paradigm was then demonstrated using GDNF-releasing PAMs, conveying a small number of embryonic ventral mesencephalon dopaminergic cells (Tatard, Sindji et al. 2007).

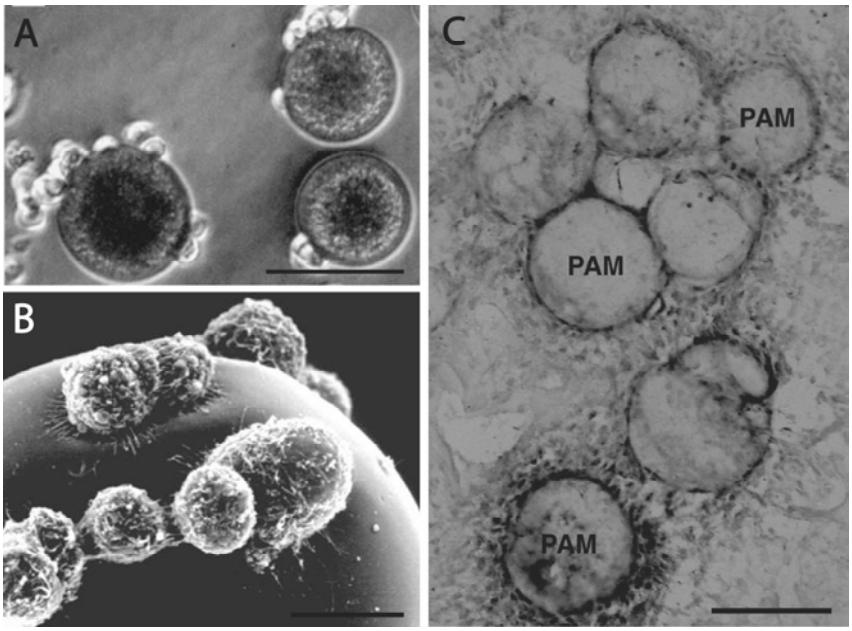

Figure 6. NGF releasing PAMs in combination with PC12 cells

PC12 cells adhered onto PAMs surface as observed by optic (A) and scanning electron microscopy (B). After transplantation in the rat brain, the NGF is released in the tissue 2 weeks after implantation and diffuses around the microcarriers. It should thus be available for most of the cells in the graft. TH immunoreactive cells are observed around the PAMs (C). Scale bars A-C: 50 μm; B: 5 μm. Abbreviations: NGF: nerve growth factor, PAM: pharmacologically active microcarrier. Adapted with permission from Tatard et al., Biomaterials, 2005.

Synergic effects between adhesion and growth factor signals to guide and enhance cell differentiation have now been described (Nakajima, Ishimuro et al. 2007). Such effects were also observed on sensory neurons which exhibited an increased neurite length when cultivated in the presence of LM and NGF *in vitro*, a cooperation that may, at least partly, be mediated via the phosphorylation of the protein Src (Tucker, Rahimtula et al. 2005). In a similar manner, a team reported the differentiation of NSCs into dopaminergic cells *in vitro*, upon culture on a LM substrate and exposure of cells to a combination of bFGF and heparin. Moreover, dopaminergic neurons were also observed *in vivo* after transplantation in a rat model of PD when this pre-treatment was used for cell priming (Yiqun Yu 2007).

To implement the idea of combining the biomimetic approach with the controlled delivery of a growth factor in the context of PD adult cell therapy, we optimized PAMs for use with the MIAMI cells. Indeed, combining growth factors, cell adhesion molecules and an adapted 3D structure in the same polymeric scaffold allows the synthesis of an adaptable and very efficient system that can deliver stem cells and give them appropriate cues allowing better stem cell survival, differentiation and integration into the host tissues after implantation.

MIAMI cells are known to differentiate toward the ectodermal lineage in a neurotrophin 3 (NT3) dependent manner (Tatard, D'Ippolito et al. 2007) and our recent studies (see section 3.3.2) demonstrated an enhanced neuronal differentiation of these cells on a LM substrate *in vitro*. Therefore, the encapsulation of NT3 inside these 60 µm PLGA microspheres, together with a LM biomimetic surface should improve the survival and neuronal differentiation of MIAMI cells after transplantation into the striatum of hemi-parkinsonian rats. In addition, encapsulated NT3 may directly affect the host microenvironment, as observed in the central nervous system with NT3-releasing fibrin scaffolds (Johnson, Parker et al. 2009). Interestingly, the potential of microsphere-hydrogel scaffolds to deliver 2 growth factors at specific rates have been described (Burdick, Ward et al. 2006), but have not yet led to *in vivo* studies. MIAMI cells in combination with PAMs encapsulating NT3 and coated with LM may become a powerful tool for PD cell therapy. To our knowledge, this is the first tissue engineering strategy combining adult cells with the biomimetic approach and the controlled delivery of a growth factor to treat PD.

4. CONCLUSION

In a general manner, therapies for brain neuronal damages are still mainly focused on alleviating the symptoms, sometimes with severe side-effects. Cell therapy, with the possibility to repair the degenerated tissue, holds tremendous promise for the treatment of neurological disorders. After a great number of preliminary studies using foetal or embryonic precursors for adult brain cell therapy, the need for an alternative cell source is now compulsory. Adult cells may alleviate ethical and availability concerns, with the additional advantages, in some cases, to allow autologous grafts to be performed. In this regard, an increasing number of studies, mainly using MSCs, have provided encouraging results in the context of several central nervous system disorders. However, the major problems of cell survival, cell fate determination and engrafment after transplantation, still remain. Indeed, understanding of the early death mechanisms (oxygen deprivation, necrosis, apoptosis and shear stress) as well as the means to ameliorate them is still required. Tissue engineering may contribute to alleviate some or all of these issues and several proofs of concept have already been reported with even some clinical trials, as in the case of Spheramine ® for PD. Advanced scaffold modifications, such as the biomimetic approach, are still in their infancy in regard to adult cells but would allow to better control cell survival, proliferation, migration, differentiation and engrafment *in vivo*. Transplantation of gel-based scaffolds populated with hMSCs is a good example of an effective cell delivery vehicle for neuroprotection and repair of neural injury after TBI. Similarly, PAMs carrying human MIAMI cells may improve their survival, differentiation and repair capacities, therefore inducing functional recovery in animal models of PD.

An emerging potential source of adult stem cells, which were first described in 2007 by 2 different teams, are the iPS cells generated from terminally differentiated somatic cells via nuclear reprogramming (Takahashi, Okita et al. 2007; Yu, Vodyanik et al. 2007). Although the methodology is still in its infancy, these cells may overcome the ethical problems linked with ES cells and can potentially be derived from any adult patient, with a predicted similar pluripotency of ES cells. Interestingly, reprogrammed fibroblasts may be induced to differentiate into RPE-like cells (Buchholz, Hikita et al. 2009; Osakada, Jin et al. 2009) and neuron-like cells; the latter being able to correct neurologic deficits in a rat model of Parkinson's disease (Wernig, Zhao et al. 2008). At present, iPS cells are obtained via retroviral or lentiviral cell transduction, this being a practical limitation for use in clinical applications because genome-integrating viral vectors may, for example, cause insertional

mutagenesis. However, with the intensive development of new protocols to avoid cell genome modifications during the induction of pluripotency (Page, Ambady et al. 2009; Yu, Hu et al. 2009; Zhou, Wu et al. 2009), these cells may in the future safely be used in the clinic.

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CHAPITRE 2

Devenir des cellules stromales mésenchymateuses *in vivo*

INTRODUCTION

Quel est le devenir des cellules stromales mésenchymateuses *in vivo*?

Ayant pour objectif la transplantation de CSM pour la thérapie du SNC, il était important de se questionner sur le devenir *in vivo* de ces cellules, à la fois en terme de survie, de différenciation, mais également de migration. Ainsi, nous souhaitions notamment vérifier si des CSM transplantées dans le striatum, mais plus particulièrement dans la zone hautement neurogénique de la ZSV en bordure du ventricule latéral, restaient au niveau du site d'injection ou si elles migraient vers d'autres zones du cerveau. En effet, peu d'études décrivent la migration de CSMs implantées dans des cerveaux sains (Phinney, Baddoo et al. 2006), alors que la capacité de migration des CSM vers des lésions est largement décrite, raison pour laquelle nous avons également réalisé une lésion du bulbe olfactif dans un groupe de rats afin de stimuler la migration des cellules transplantées.

Des CSN furent utilisées en tant que contrôle positif pour le suivi de cette éventuelle migration. En effet les CSN, d'origines murines mais également humaines, possèdent une capacité de migration intrinsèque de la ZSV vers le bulbe olfactif de cerveau de rats adultes (Fricker, Carpenter et al. 1999; Englund, Fricker-Gates et al. 2002). Ainsi, une étude a montré qu'une importante migration était observée après implantation directe de CSN dans le courant de migration rostral alors qu'une faible migration était observée après transplantation au cœur du striatum (Cicchetti, Gross et al. 2007). Des cellules de rats furent utilisées dans cette étude pour des raisons de disponibilité des cellules humaines, mais aussi afin d'éviter leur rejet ainsi que pour pallier à la lourdeur des protocoles d'immunosuppression.

Les technologies nanoparticulaires peuvent avoir de multiples applications dans le cadre du SNC (Provenzale and Silva 2009), et peuvent notamment permettre de suivre la migration de cellules. Ainsi, nous avons opté pour un marquage des cellules par des nanoparticules d'oxyde de fer enrobées de bisphosphonates (1-hydroxyethylidene-1.1-bisphosphonic acid, HEDP) développées au laboratoire (Portet, Denizot et al. 2001). Après absorption par les cellules et transplantation *in vivo*, ces nanoparticules provoquent un hypo-contraste visualisable de façon non-invasive par IRM. De plus, la surface d'HEDP offre la possibilité de fonctionnaliser ces nanoparticules, augmentant ainsi le champ des applications éventuelles par ciblage spécifique des cellules marquées. La haute résolution de cette technique peut permettre un suivi non-invasif à l'échelle cellulaire (Heyn, Ronald et al. 2006) et permet de réduire significativement le nombre d'animaux de laboratoire nécessaire aux expérimentations. De plus, elles permettront peut-être dans le futur un suivi des cellules

greffées au cours d'essais cliniques de thérapie cellulaire. De nombreux types cellulaires, y compris les CSN (Cicchetti, Gross et al. 2007; Guzman, Uchida et al. 2007), ont déjà été marqués et suivis avec succès par des nanoparticules d'oxyde de fer (Bulte, Duncan et al. 2002; Modo, Cash et al. 2002; Sykova and Jendelova 2007b). Cependant, l'innocuité des nanoparticules sur les cellules greffées ainsi que sur leur comportement *in vitro,* mais également *in vivo,* est encore débattu (Omidkhoda, Mozdarani et al. 2007). Afin d'améliorer l'absorption cellulaire de ces nanoparticules et d'en diminuer la toxicité, de nombreuses recherches se concentrent sur l'utilisation d'agents facilitant l'incorporation (Arbab, Bashaw et al. 2003) comme la lipofectamine (Frank, Miller et al. 2003), ou sur la conjugaison des nanoparticules avec le peptide viral Tat (Lewin, Carlesso et al. 2000). Enfin, des anticorps facilitant l'internalisation des nanoparticules sont également parfois utilisés (Bulte, Zhang et al. 1999). Ces méthodes restent toutefois controversées (Arbab, Jordan et al. 2004) et l'utilisation d'agents de transfection pourrait ne pas être autorisée en clinique (Mailander, Lorenz et al. 2008).

Dans cette étude, nous nous sommes donc efforcés de minimiser la toxicité des nanoparticules utilisées tout en maximisant l'efficacité du marquage cellulaire, sans ajout d'agents facilitants. Nous nous sommes ensuite attachés à confirmer l'intégrité de leur potentiel de différenciation *in vitro* avant de les suivre *in vivo*. Le suivi par IRM a été complété par un marquage histochimique appelé « bleu de Prusse », spécifique des nanoparticules d'oxyde de fer, ainsi que par un marquage préalable des cellules au BrdU, un analogue de la thymidine s'incorporant à l'ADN des cellules en division.

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Research Report

Mesenchymal and neural stem cells labeled with HEDP-coated SPIO nanoparticles: In vitro characterization and migration potential in rat brain☆

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ABSTRACT

Mesenchymal stem cells°MSC) may transdifferentiate into neural cells in vitro under the influence of matrix molecules and growth factors present in neurogenic niches. However, further experiments on the behavior of such stem cells remain to be done in vivo. In this study, rat MSC (rMSC) have been grafted in a neurogenic environment of the rat brain, the subventricular zone (SVZ), in order to detect and follow their migration using superparamagnetic iron oxide (SPIO) nanoparticles. We sought to characterize the potential effect of iron loading on the behavior of rMSC as well as to address the potential of rMSC to migrate when exposed to the adequate brain microenvironment. 1-hydroxyethylidene-1.1-bisphosphonic acid (HEDP)-coated SPIO nanoparticles efficiently labeled rMSC without significant adverse effects on cell viability and on the in vitro differentiation potential. In opposition to iron-labeled rat neural stem cells (rNSC), used as a positive control, iron-labeled rMSC did not respond to the SVZ microenvironment in vivo and did not migrate, unless a mechanical lesion of the olfactory bulb was performed. This confirmed the known potential of iron-labeled rMSC to migrate toward lesions and, as far as we know, this is the first study describing such a long distance migration from the SVZ toward the olfactory bulb through the rostral migratory stream (RMS).

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Abbreviations: BrdU, bromodeoxyuridine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate buffered saline; EDX, energy dispersive X-ray; FBS, foetal bovine serum; HEDP, 1-hydroxyethylidene-1.1-bisphosphonic acid; HG, high glucose; IHC, immunohistochemistry; LG, low glucose; MIAMI, marrow isolated adult multilineage inducible; MRI, magnetic resonance imaging; MSC, mesenchymal stem cell; OB, olfactory bulb; PB, Prussian blue; PFA, paraformaldehyde; RMS, rostral migratory stream; rMSC, rat mesenchymal stem cell; rNSC, rat neural stem cell; RT, room temperature; SEM, scanning electron microscopy; SPIO, superparamagnetic iron oxide; SVZ, subventricular zone; TEM, transmission electron microscopy

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1. Introduction

Stem cells are often described as the best candidates for cell therapy studies due to their self-renewal capacity and their large differentiation potential. Among them, mesenchymal stem cells (MSC) remain easy to isolate and expand. They may exhibit immunodepressive characteristics which make them less sensitive to rejection by the host immune system (Le Blanc et al., 2003; Maitra et al., 2004; Nasef et al., 2007). Moreover, MSC allow autologous grafts to be performed in cell therapy protocols. Bone marrow MSC typically differentiate into connective tissue cell types (D'Ippolito et al., 2004; Jiang et al., 2002), but various laboratories have also reported the transdifferentiation potential of MSC into a neuronal-like phenotype (Black and Woodbury, 2001; Sanchez-Ramos et al., 2000; Trzaska et al., 2007). We previously showed that a subpopulation of human MSC, marrow-isolated adult multilineage inducible (MIAMI) cells may trandifferentiate in vitro in a neurotrophin-dependent manner into neuronal-like cells. These cells express neuronal markers and present electrophysiological characteristics similar to those observed in mature neurons (Tatard et al., 2007). Moreover, a fraction of MSC transplanted in adult rat brains may respond to microenvironmental cues and transdifferentiate into neuronal-like cells (Jendelova et al., 2004; Kopen et al., 1999; Zhao et al., 2002). Using different brain lesion models, it has been shown that implanted MSC may be involved in functional improvement, either directly or indirectly by their ability to produce various growth factors (Chen et al., 2002; Li et al., 2002; Mahmood et al., 2002; Zhang et al., 2005). In addition, a damaged environment resulting e.g. from ischemia or from the presence of a tumor is known to stimulate the migration of transplanted MSC (Jendelova et al., 2004; Mahmood et al., 2002; Sykova and Jendelova, 2007a) as well as of neuronal precursors (Aboody et al., 2000; Kokaia and Lindvall, 2003). MSC may thus be considered as potential candidates for cell therapy studies in the central nervous system.

However, the possible use of MSC for brain repair studies still requires an evaluation of their behavior, migratory dynamic and fate in vivo. Moreover, as only a fraction of the transplanted cells may respond to the stimuli of the

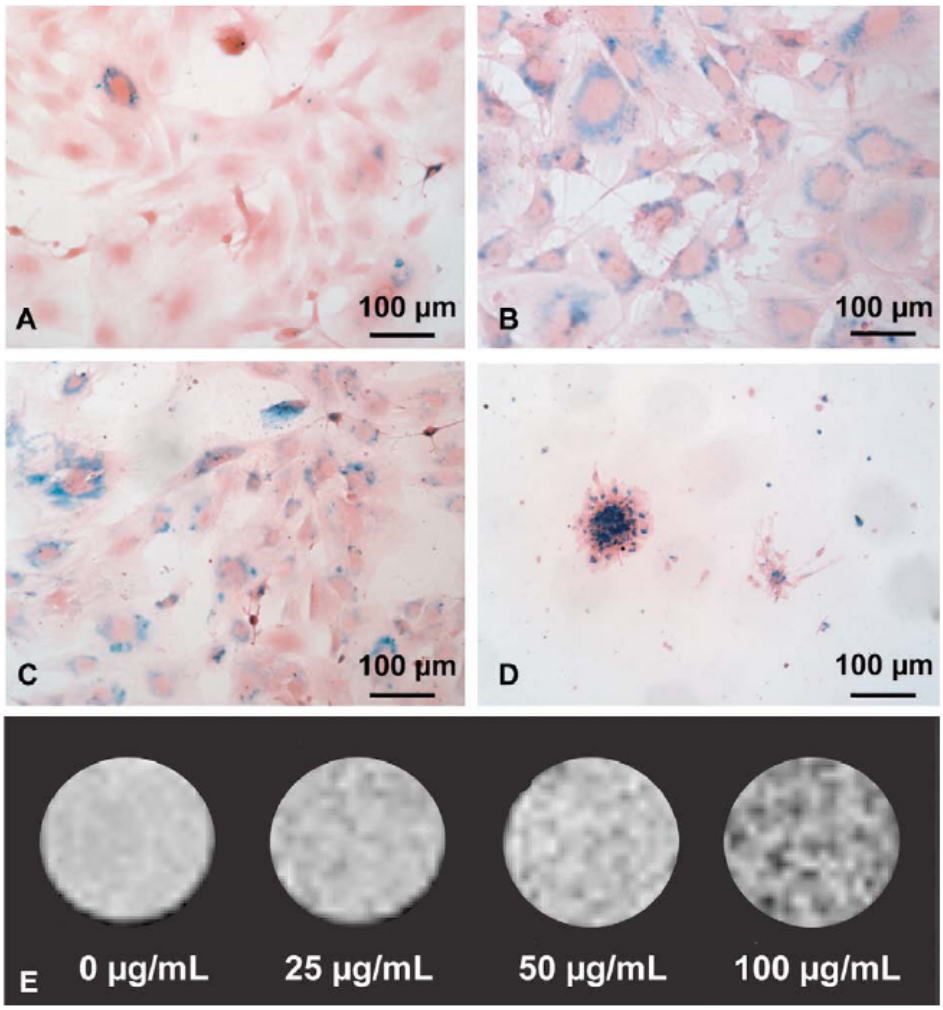

Fig. 1 – In vitro Prussian blue assessment of iron uptake and MRI. The intensity and the percentage of PB positive cells (in blue) increased with the iron concentration used. rMSC incubated for 48 h with 25, 50 and 100 µg/mL iron (A, B and C respectively). rNSC incubated for 24 h with 50 µg/mL iron under adherent conditions (D). In vitro T2*-weighted images (TE = 15 ms) of an agarose gel containing rMSC incubated for 48 h in culture medium containing HEDP-coated nanoparticles (E). From left to right, iron concentration in the media was 0, 25, 50 and 100 µg/ml.

Table 1 – Spectroscopic iron titration

Iron concentration used for incubation (μg/mL)	Iron content (pg/cell)
25	3.5±2.3
50	5.6±1.6
100	9.9±3.8

Average iron concentration per rMSC increased with the iron concentration used (from ca. 4 to 10 pg/cell) after 48 h incubation.

microenvironment, highly sensitive procedures will be required in the future.

Magnetic resonance imaging (MRI) is a non-invasive tool that has demonstrated a high sensitivity for cell tracking after systemic or in situ injection of cells having incorporated magnetic tags. Indeed, using phagocytic cells, the detection of a single cell in mouse brain has been obtained with this technique (Heyn et al., 2006). This strategy spares laboratory animals and ultimately may be used for human stem cell therapy studies. Toward this end, new magnetic tracers readily and specifically taken up by stem cells need to be formulated. Superparamagnetic iron oxide (SPIO) nanoparticles stand as promising tools to label and track various cell types in vivo by MRI (Bulte et al., 2002; Modo et al., 2002; Sykova and Jendelova, 2007b). We developed SPIO nanoparticles coated with 1-hydroxyethylidene-1.1-bisphosphonic acid (HEDP) (Portet et al., 2001), which are interesting due to their possible functionalization allowing the targeting and uptake of a specific cell type. In order to be able to translate this tool into the clinic, the innocuousness and the non-interference of these nanoparticles with the response of stem cells to their microenvironment have to be demonstrated.

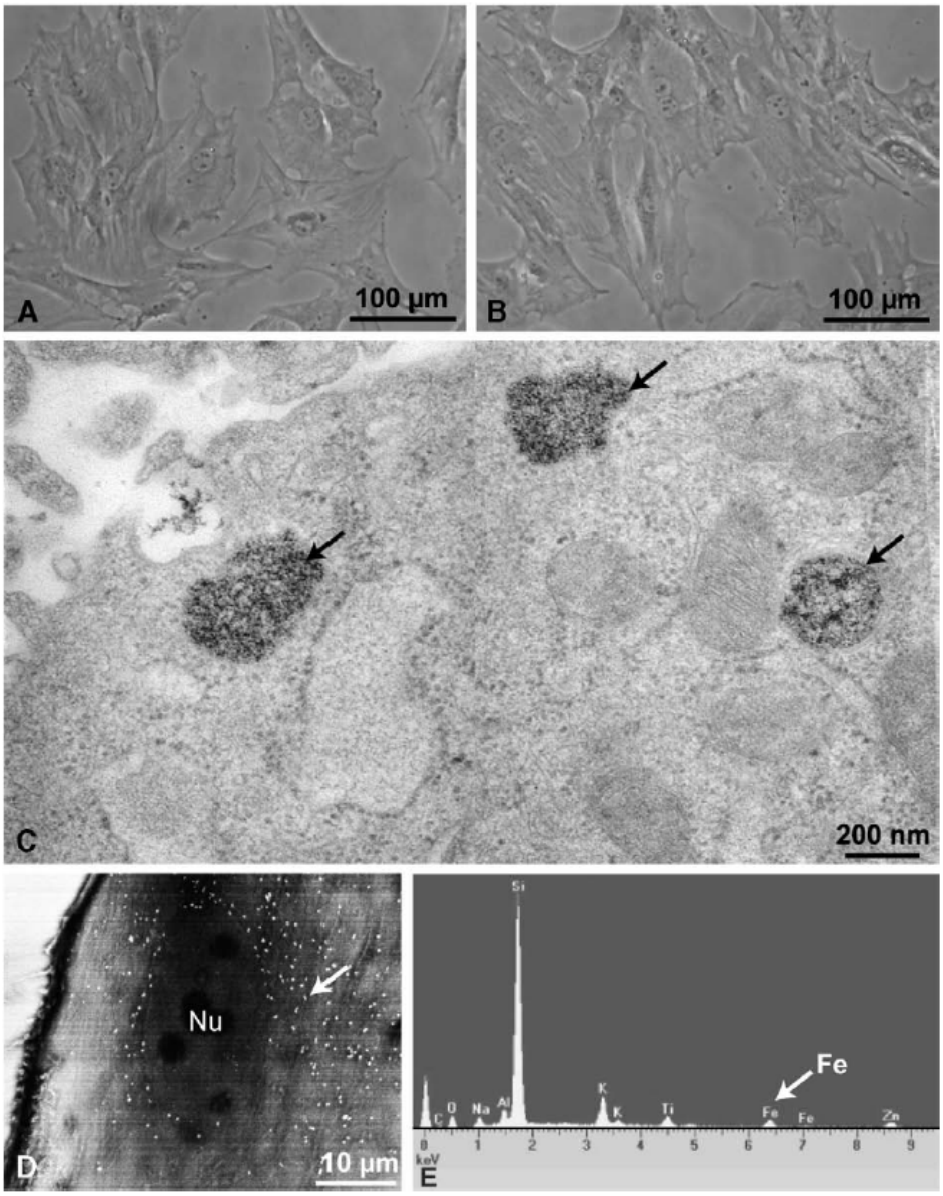

Fig. 2 – Iron-labeled rMSC microscopical analysis. Morphology of unlabeled rMSC (A) was identical to rMSC labeled with 50 μg iron/mL during 48 h (B). TEM pictures showing the endo-lysosomial localization of the nanoparticles, iron in black (C). Back-scattered SEM picture (11 kV), iron in white (D). EDX spectrum confirming the iron nature of the white dots observed by SEM (E). Nu: nucleus.

In this study, we sought to characterize the potential effects of labeling rat MSC (rMSC) with these HEDP-coated SPIO nanoparticles on their viability and functions in vitro. Toward this end, rMSC iron uptake was characterized in vitro by Prussian blue (PB) staining and MRI. Iron-labeled rMSC viability and ultrastructure were also studied, as well as their osteogenic and neuronal differentiation potentials. Furthermore, we assessed the migratory potential of these iron-labeled MSC in vivo in response to the brain neurogenic stimuli. Indeed, it has been shown that neural stem cells (NSC) migrated from the subventricular zone (SVZ) to the olfactory bulb (OB), via the rostral migratory stream (RMS), where they differentiate into post-mitotic interneurons (Coskun and Luskin, 2002). Therefore, we studied the ability of iron-labeled rMSC to migrate in a similar fashion than iron-labeled rat neural stem cells (rNSC), when transplanted into the SVZ of the lesioned or non-lesioned rat brain. This study was performed by bromodeoxyuridine (BrdU) immunohistochemistry (IHC) and PB staining. Finally, a double staining with PB/CD11b, specific for macrophage/microglia, was used to confirm the fate of the grafted cells and of the SPIO nanoparticles.

2. Results

2.1. *Iron uptake and cell characterization*

Prussian blue staining, after incubation of the rMSC with the iron-oxide nanoparticles, demonstrated that the intensity and the percentage of labeled cells increased with the iron concentration used. Forty eight hours incubation with an amount of nanoparticles corresponding to 25 μg iron/mL resulted in ca. 10% of Prussian blue positive cells, 50 μg/mL efficiently labeled more than 90% of the cells whereas 100 μg iron/mL did not increase the percentage of positive cells compared to 50 μg iron/mL (Figs. 1A and B, C respectively). For rNSC, PB staining revealed that 24 h of incubation with 50 μg/mL iron gave the most efficient labeling (Fig. 1D). rNSC were expanded as floating clusters called neurospheres but incubation in the iron containing medium was performed on adherent progenitors as the 3D structure of the neurospheres prevented an efficient labeling of all the cells.

Spectroscopic iron titration confirmed that the iron content per rMSC varied with the nanoparticle concentration used during labeling. Over the concentration range used (from 25 to 100 μg iron/mL), the intracellular concentration increased from ca. 3.5 pg/cell to 10 pg/cell (Table 1). The differences in iron content per cell were also observed using in vitro MRI. Indeed, Fig. 1E shows typical T2*-weighted images of an agarose gel containing iron-labeled rMSC. As the iron concentration increased in the incubation media, the T2* effects induced by the cells increased. Thus, cells incubated with 50 μg iron/mL induced a global T2* reduction of ca. 50% compared to unlabeled cells.

Trypan blue counting and MTS assay showed that cell viability was not affected one day post-incubation when using 25 or 50 μg iron/mL, whereas it decreased to 70% of control with 100 μg iron/mL. Consequently, 50 μg iron/mL incubated for 48 h was chosen as the best trade-off in terms of iron content, percentage of marked cells and cell viability. It resulted in an average concentration of 5.6±1.6 pg iron/cell as determined by spectroscopic iron titration (Table 1). It should be noted that the facilitating Fugene® reagent was tested but did not significantly enhance iron uptake (data not shown). It was consequently not used in this study.

Under these conditions, iron-labeled cells did not exhibit morphological differences with unlabeled cells (Figs. 2A and B). Moreover, transmission electron microscopy (TEM) demonstrated that iron-labeling did not affect rMSC ultrastructure and that the SPIO nanoparticles were taken into the cells rather than adhering to the exterior of the membrane. They were located in endo-lysosomial vesicles with a mean diameter of 355 nm (Fig. 2C). Observations by scanning electron microscopy (SEM) confirmed the intracytoplasmic and perinuclear distribution of the nanoparticles. The small white dots observed on back-scattered SEM images (Fig. 2D) were correlated to a difference in molecular composition and an energy dispersive X-ray (EDX) spectrum confirmed their iron nature (Fig. 2E).

To confirm that iron-labeled rMSC maintain their function in vitro, differentiation studies were performed. Iron-labeled rMSC were still able to undergo an osteogenic differentiation. Moreover, a similar proportion of cells responded to this induction when compared to the control, unlabeled cells, as shown by Alizarin red staining of hydroxyapatite associated calcium mineral deposits (Figs. 3A and B). In addition, rMSC developed features of neuronal cells in response to published protocols (Woodbury et al., 2000). Most importantly, there was no significant difference between unlabeled and iron-labeled rMSC responses to the neuronal induction in vitro. Flat, uninduced rMSC expressed low levels of nestin and β3-tubulin (Table 2). When exposed to the neuronal induction medium, some cells detached but most of them exhibited a narrower morphology that evolved towards a neuronal-like morphology, ranging from simple bipolar to branched cells (Figs. 3C to H). Moreover, rMSC with a neuronal-like morphology acquired the expression of the neural-related markers nestin and β3-tubulin. Up to 50% and 65% of rMSC were positive for nestin and β3-tubulin, respectively, 24 h post-induction. β3-tubulin expression remained stable 48 h post-induction whereas nestin expression decreased to 5–35% positive cells (Table 2). The neuronal marker NeuN was already expressed in expanding cells and at a similar level at every step of the neuronal induction (Figs. 3G and H).

2.2. *Labeling for histochemical detection*

Different in vitro labeling techniques (Hoechst 33342, PKH26, GFP transduction and BrdU) were tested to co-localize the iron-labeled cells after transplantation and determine their fate in vivo. PB staining of the labeled cells in vitro demonstrated that none of the methods used significantly interfered with iron uptake. However, some of these techniques were not completely satisfactory. Co-culture experiments with GFP-positive rMSC and Hoechst 33342-positive rMSC demonstrated that Hoechst fluorescent dye diffused into GFP-positive rMSC. PKH26 was readily absorbed by the rMSC and did not diffuse to the surrounding cells but the correct visualization of the cells was impaired in vivo due to a weak fluorescent signal.

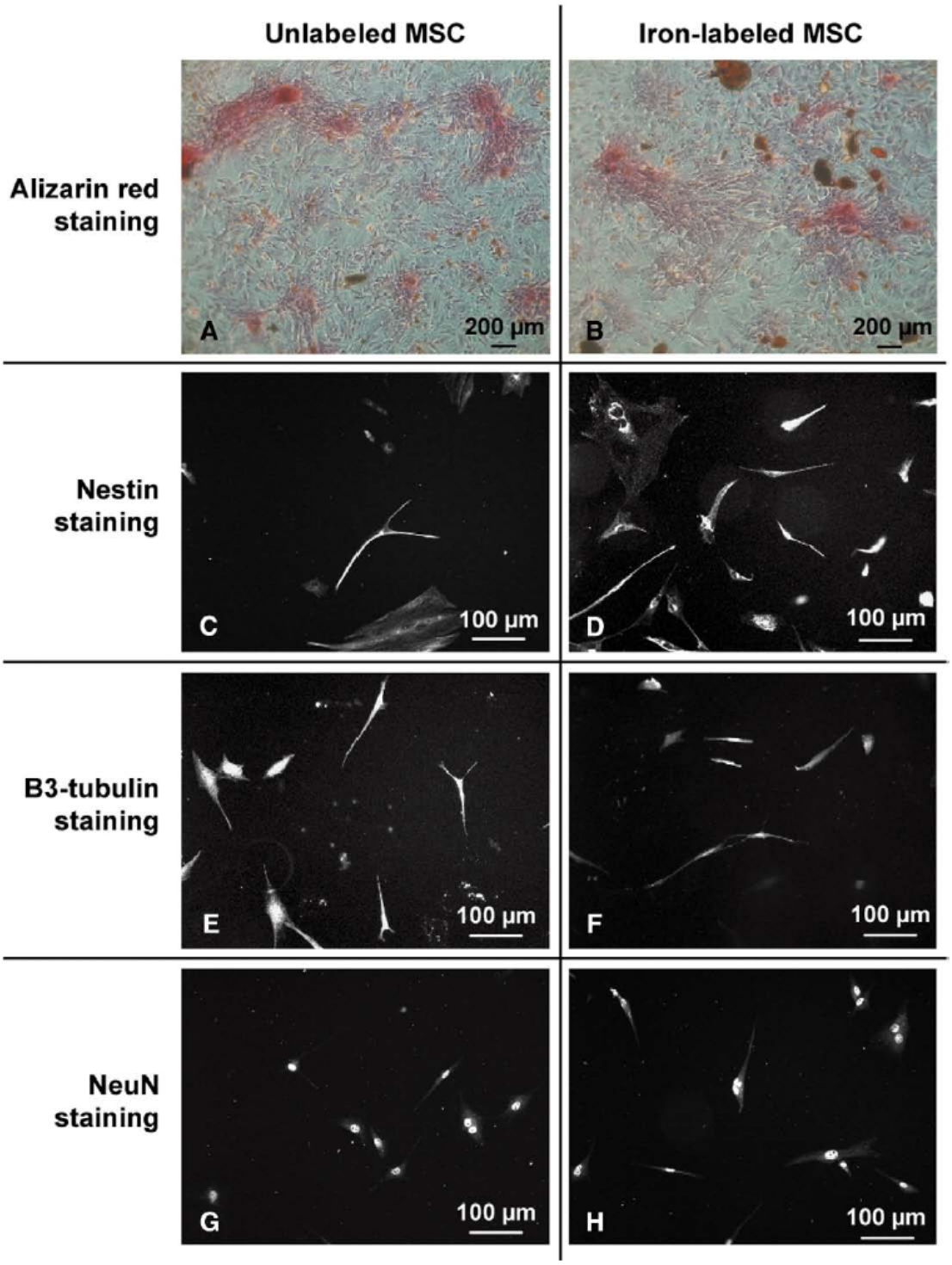

Fig. 3 – rMSC in vitro differentiation. When induced toward the osteogenic lineage, unlabeled rMSC (A) as well as iron-labeled rMSC (B) produced hydroxyapatite calcium mineral deposits that stained red with Alizarin. After treatment with the neuronal induction media, flat rMSC acquired a narrower, spindle shaped morphology and exhibited an increased expression of the neural-related markers nestin and β3-tubulin. The neuronal marker NeuN was already expressed in expanding cells and at every steps of the neuronal induction. Most importantly, unlabeled and iron-labeled rMSC responded similarly to the neuronal induction. Nestin, β3-tubulin and NeuN expression 48 h post-induction was identical for unlabeled (C, E and G respectively) and iron-labeled rMSC (D, F and H respectively).

Lentiviral transduction yield of GFP was around 80% but resulted in a decreased cell proliferation rate, as well as a lower response of the cells to neuronal induction in vitro (data not shown).

Cell detection by PB staining and BrdU IHC was the most suitable labeling strategy, in our hands, to study the in vivo distribution of iron-labeled rMSC in rat brains. A minimum incubation time of 72 h was used to label 90% of the iron-

Table 2 – rMSC in vitro neuronal induction

		Uninduced rMSC	24 h post-induction	48 h post-induction
% Nestin positive	Unlabeled	5–15%	25–50%	5–30%
	Iron-labeled	5–15%	25–50%	10–35%
% β3-tubulin positive	Unlabeled	0–10%	15–55%	15–50%
	Iron-labeled	0–5%	15–65%	25–50%

Unlabeled and iron-labeled rMSC responded similarly to the in vitro neuronal induction protocol. Percentage of nestin positive rMSC increased up to 50% 24 h after induction and decreased 48 h post-induction. Percentage of β3-tubulin positive rMSC increased up to 65% 24 h after induction and remained stable 48 h post-induction.

labeled rMSC in vitro with BrdU (Fig. 4A). Indeed, an incubation time of 48 h resulted in only 40% of BrdU positive rMSC (Fig. 4B). The rapidly dividing iron-labeled rNSC were efficiently labeled with BrdU after only 6 h incubation (Fig. 4C). These incubation times were then used to load the cells with BrdU prior to transplantation.

2.3. *In vivo detection of rNSC and rMSC*

In the non-lesioned brain model, rMSC grafted into the SVZ did not respond to the stimuli of the neurogenic micro-environment and were only found in the grafting site (Fig. 5A). Preliminary results indicated that, 5 days after grafting, rMSC close to the injection site stained positive for nestin as well as slightly for β3-tubulin (data not shown). We demonstrated that a mechanical lesion of the OB strongly induced the migration of rMSC to the OB through the RMS. This migratory behavior was confirmed by the visualization of the cells on adjacent brain slices both by BrdU IHC and PB staining 4, 14 and 21 days after transplantation, with a fainter staining after 21 days. The pathway followed by the cells from the injection site to the OB was approximately 7 mm long. Time after grafting was a critical parameter as only a few migrating cells were observed 4 days after transplantation whereas the highest number was observed after 14 days. A fraction of cells were observed near the injection tract, but also all along the RMS and many of them reached the OB and were dispersed in this region (Fig. 5 and Table 3). Prussian blue staining performed on control rats was negative; therefore confirming that PB staining in the grafting site as well as in the lesioned OB was specific for iron-labeled cells and did not result from endogenous iron deposit. No strong inflammatory reaction was observed with CD11b staining, a specific marker for macrophage/microglia. Moreover, PB/CD11b double staining revealed a co-localization of this marker with only a few PB-positive cells, but not with the entire PB-positive cell population (Figs. 5K and L).

However, even though PB staining revealed a large number of iron-labeled rMSC in the lesioned OB region after the mechanical lesion (Fig. 5D), this pool of migrating cells was not accessible to MRI as the lesion itself was responsible for a large hyposignal and labeled cells were only efficiently visualized at the grafting site (Figs. 6A and B).

In non-lesioned rat brains, rNSC were found in the grafting site, in the RMS and in the OB 14 days after transplantation (Figs. 5F and G and Table 3). Thus, iron-labeled rNSC migrated

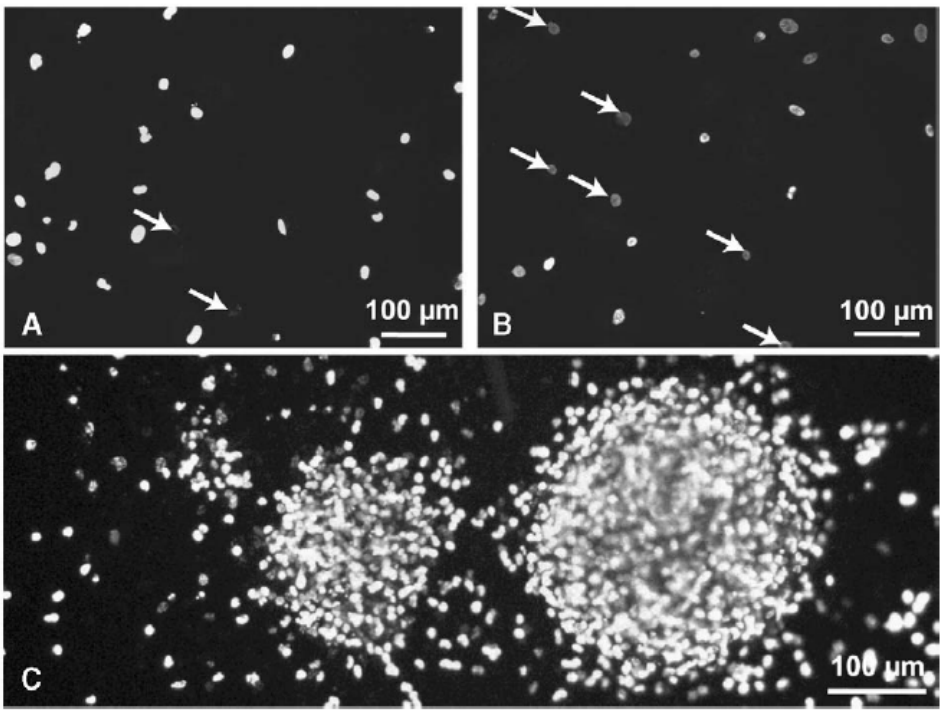

Fig. 4 – BrdU immunofluorescent staining of iron-labeled rMSC and rNSC. Ninety percent of rMSC were BrdU positive after 72 h incubation (A) whereas only 40% were positive after 48 h (B) (white arrowheads point out BrdU negative cells). Six hours incubation under adherent conditions was sufficient to label rNSC (C).

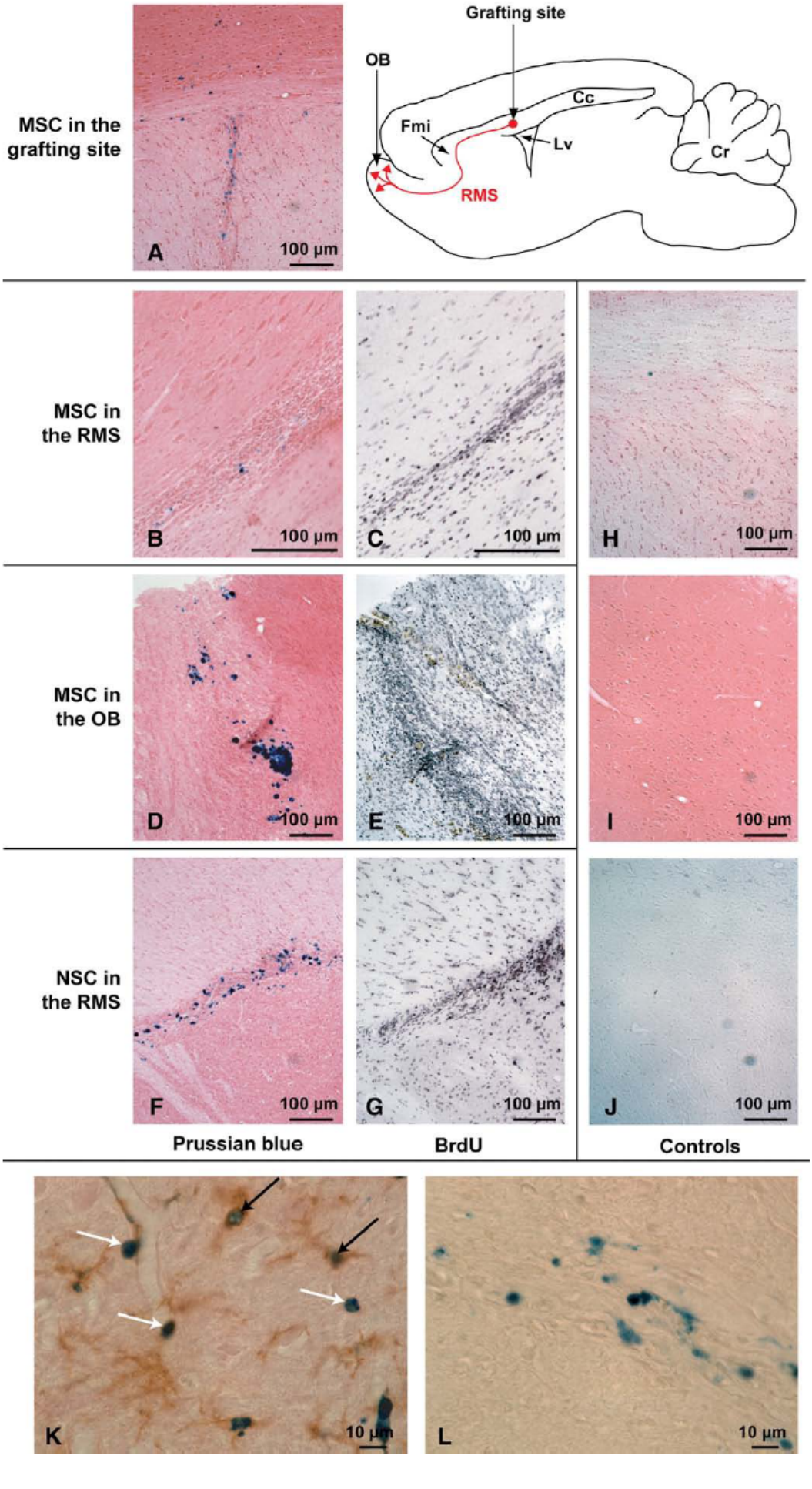
MSC in the grafting site
Grafting site
OB
Cc
Fmi
Lv
Cr
RMS
A
100 µm
MSC in the RMS
B
C
H
MSC in the OB
D
E
I
NSC in the RMS
F
G
J
Prussian blue
BrdU
Controls
K
L
10 µm

Table 3 – Cell migration assessment by PB staining and BrdU staining

Cell type	Time after grafting (days)	Transplanted cell number	Lesioned OB	Labels	SVZ	RMS	OB
rNSC	14	80000	–	BrdU	+	+	+
				PB	+	++	–
rMSC	4	100000	+	BrdU	+/–	+/–	+/–
	14	80000	–	PB	+	–	–
	14	60000/80000	+	BrdU	+/–	+	+
				PB	+[1]	+[1]	++[2]
	21	40000	+	BrdU	+/–	+/–	–
				PB	+	+/–	–

Iron-labeled rNSC migrated toward the OB after grafting into the SVZ whereas iron-labeled rMSC did not, unless a mechanical trauma of the OB was performed. In this condition, less than 5 PB positive rMSC per slides were detected in the grafting site and in the RMS[1], and approximately 90±50 cells in the OB[2] 14 days after transplantation (mean of 3 slides±standard deviation).

in a similar way than endogeneous rNSC in the absence of lesion.

3. Discussion

Potential adverse effects of iron nanoparticles is a topic currently under investigation (Omidkhoda et al., 2007), and the means to diminish this side effect still remain to be explored. Many authors described the use of facilitating agents (Arbab et al., 2003) such as lipofectamin (Frank et al., 2003), conjugation with Tat peptide (Lewin et al., 2000) or with internalizing antibodies (Bulte et al., 1999) to increase the uptake efficiency while diminishing cell toxicity. However, these methods are also currently under debate (Arbab et al., 2004) and transfecting agents may not be approved for clinical use (Mailander et al., 2008). In our study, we demonstrated an efficient and non toxic in vitro uptake of native HEDP-coated SPIO nanoparticles by rMSC without using any transfecting agent. In addition, the bisphosphonate (HEDP) coating offers the possibility to functionalize the nanoparticles, therefore increasing the range of potential applications.

Forty-eight hours incubation with 50 μg iron/mL of these SPIO nanoparticles was the best trade-off in terms of labeling efficiency and cell viability. Under the conditions used, more than 90% of the rMSC contained enough iron allowing their detection with PB staining; amount that is in the same range than what is usually described e.g. with D-mannose-coated nanoparticles where 80% of the cells were labeled (Sykova and Jendelova, 2007b). An efficient uptake is indeed required as a simple adhesion on the cell surface could be detrimental for the cell response to the microenvironment in vivo (Sykova and Jendelova, 2007b). In this regard, we confirmed the endocytosis of the iron nanoparticles by SEM and TEM studies. In our study, the average iron content per cell (5.6±1.6 pg) was low compared to what is described with polycation-bound SPIO nanoparticles (38 pg iron/cell) (Sykova and Jendelova, 2007b), but in the same range to what is obtained when labeling human MSC with the ferucarbotran Resovist® (Mailander et al., 2008).

In addition to an absence of acute toxicity, the HEDP-coated nanoparticles had no significant side effects on rMSC behavior in vitro. The ultrastructure was conserved and their differentiation potential toward the osteogenic as well as neuronal lineages did not exhibit significant differences with unlabeled rMSC. MSC differentiation toward a neuronal-like lineage is now well-documented (Black and Woodbury, 2001; Sanchez-Ramos et al., 2000; Tatard et al., 2007; Trzaska et al., 2007) and the fraction of differentiating rMSC (50 to 65%) confirmed results published with this protocol (Woodbury et al., 2000). Interestingly, NeuN was always detected in the rMSC used in this study. In this regard, the expression of neuronal markers in expanding, uninduced MSC has already been described (Tondreau et al., 2004). These results complement previous studies which described a conserved adipogenic and osteogenic differentiation potential (Arbab et al., 2005; Farrel et al., 2008) even if chondrogenic differentiation was sometimes impaired with Feridex® nanoparticles (Kostura et al., 2004). Altogether, these results confirmed the possible use of the HEDP-coated nanoparticles for cell tracking as it does not seem to interfere with the in vitro rMSC biology. Moreover, trials made to label a subpopulation of human MSC, the MIAMI cells (D'Ippolito et al., 2004) showed that they readily absorbed HEDP-coated iron nanoparticles, even with a higher efficiency than rMSC (data not shown). As MIAMI cells present a large differentiation potential, including the ectodermal lineage, this may render possible their future use for MRI tracking in human clinical cell therapy applications (Tatard et al., 2007).

In vivo, a double staining PB/CD11b (specific for macrophage/microglia) demonstrated that CD11b-positive cells did

Fig. 5 – rMSC migratory potential assessment by BrdU immunochemistry and PB staining 14 days after transplantation. Schematic cell migration is presented on top right corner. Grafted rMSC were detected in the grafting site (A), in the RMS (B, C) and in the OB (D, E) after grafting into the SVZ. rNSC migrated from the SVZ to the OB through the RMS (F, G). In sham-operated rats, injected with medium only, no PB staining was detected in the grafting site and in the OB (H and I respectively). The BrdU isotypic control shows no background staining (J). In the grafting site, a fraction of Prussian blue positive cells co-localize with CD11b (K, black arrowheads), but not the entire rMSC population (K, white arrowheads). IgG2a isotype/Prussian blue staining shows no background (L). Cc: corpus callosum, Cr: cerebellum, Fmi: forceps minor corpus callosum, Lv: lateral ventricule.

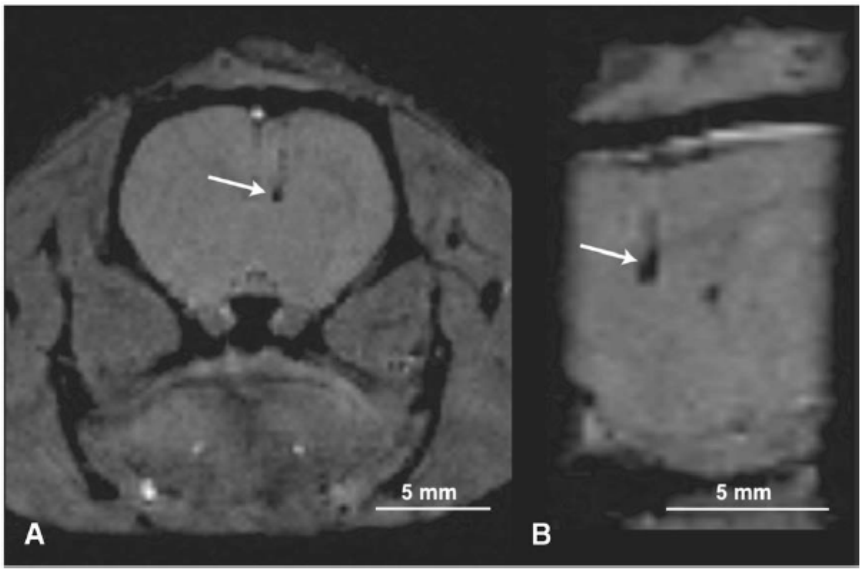

Fig. 6 – MRI visualization of grafted cells. A focal deposit of rMSC was clearly detected at the grafting site 14 days after transplantation on axial (A) and sagittal (B) planes (white arrowheads).

not, for the most part, co-localize with PB staining and therefore gave the proof that uptake by the host immune system is limited. Similar results with PB/ED1 double staining were obtained with rMSC containing dextran-coated Endorem® nanoparticles in a spinal cord injury model (Sykova and Jendelova, 2007a). To our knowledge, this phenomenon has not been previously described in other cell types, suggesting that this event may be cell dependent. Therefore, the fate of iron nanoparticles will have to be addressed in more details in future studies using MSC. On the other hand, a possible transfer of BrdU to the host cells 3 weeks after transplantation has been described (Burns et al., 2006), but as BrdU labeling co-localized nicely with PB in our study, it suggests that the majority of detected cells were iron-containing rMSC and not host cells.

Iron-labeled rMSC did not respond to the microenvironmental stimuli when stereotactically implanted into the SVZ and did not migrate toward the OB via the RMS. However, when transplanted in the SVZ of a rat brain presenting a mechanical injury of the OB, the iron-labeled rMSC extensively migrated from the grafting site to the OB through the RMS, therefore covering a distance of approximately 7 mm. Indeed, rMSC were easily detected by BrdU IHC and PB staining all over the migratory pathway 14 days post-transplantation when a sufficient amount of cells ($80x10^3$) was injected. The fainter staining observed by IHC after 21 days may result from a possible cell proliferation, and therefore from an iron dilution, as was described for MSC transplanted in a brain infarct model 3 months after transplantation (Yano et al., 2005). Using MRI, the fraction of rMSC migrating toward the OB was not detected as the mechanical lesion induced a very important background hyposignal. Therefore, precautions will have to be taken in studies seeking to visualize iron-labeled cells in similar lesioned environments. It is of importance to note that we did not observe any adverse effect on the behaviour of the animals even 21 days post-transplantation. Moreover, no signs of toxicity were detected by use of current histological staining (hematoxilin/eosin), therefore confirming the safety of SPIO nanoparticles for in vivo applications (Muldoon et al., 2005).

Taken together, these in vivo results demonstrate that rMSC were not able to migrate in a similar fashion than rNSC when transplanted into the SVZ of a normal brain, but did confirm their known potential to be attracted by lesions, even after iron-loading (Jendelova et al., 2004; Sykova and Jendelova, 2005; Yano et al., 2005). It underlines the importance of the microenvironment on rMSC migratory behavior. Indeed, without lesion, in opposition to rMSC, neurosphere-derived iron-labeled rNSC, used as a positive control for cell migration, did respond to the SVZ niche stimuli and demonstrate an important migratory potential toward the OB through the RMS. This confirmed previous studies using labeled rat NSC (Cicchetti et al., 2007), unlabeled NSC (Englund et al., 2002; Fricker et al., 1999) as well as labeled human NSC (Guzman et al., 2007). The migratory response of MSC to appropriate adhesion or inflammatory molecules and to chemokines present in damaged tissues is now well documented (Ozaki et al., 2007; Spaeth et al., 2008). Indeed, many chemoattractive molecules present in a lesioned environment, including for example the monocyte chemoattractant protein-1 (MCP-1), the macrophage inflammatory protein 1α (MIP-1α) and IL-8 (Wang et al., 2002), are known to promote cell migration via an interaction with MSC cell surface receptors. Therefore, we assumed it was the underlying mechanism leading the migration of rMSC observed in the presence of an OB lesion. However, the migration in a normal adult brain of a MSC subpopulation expressing receptors known to regulate neural cell activity and migration in brain has been reported (Phinney et al., 2006). However, MSC are a heterogenous population so these receptors, including Neo1, Nrp2 and Robo1 and 4, may not be present on the rMSC used in this study as we did not observe any migration in an undamaged brain. Similarly to Phinney et al. (Phinney et al., 2006), we may hypothesize that MSC migrating in an inflammatory

environment are a different subpopulation from those that migrate in response to guidance cues modulating neural cell migration.

As a conclusion, HEDP-coated SPIO nanoparticles were used to efficiently label rMSC without impairing cell viability and structure, as well as their differentiation potentials toward the osteogenic and neuronal lineages. In vivo, iron-labeled rMSC migration toward the OB was not induced by the SVZ microenvironment. However, their well-known capacity to migrate toward lesions was confirmed as they extensively migrated toward a mechanically injured OB. Moreover, a double staining for macrophage/microglia and iron-containing cells confirmed that the majority of detected cells were not host cells. To the best of our knowledge, this was the first time that a long distance migration from the SVZ toward a lesioned OB through the RMS was described for iron-labeled rMSC. This conserved ability to migrate in vivo when stimulated by a lesion, in addition to their differentiation potential, underlines once again the possible benefits of using mesenchymal stem cells for brain cell therapy.

4. Experimental procedures

All animal experiments were conducted in accordance with the "Direction des Services Vétérinaires", the "Ministère de l'Agriculture" of France and with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

4.1. *Isolation and culture of MSC*

To obtain rMSC, tibias and femurs of 250 g female Sprague–Dawley rats (Charles River, l'Arbresle, France) were dissected. Bone marrow was harvested by flushing the medullar cavities using a 26-gauge needle with 4 ml of Dulbecco's modified Eagle's medium high glucose (DMEM-HG) (Lonza, Levallois-Perret, France) containing 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B, Sigma, St Quentin Fallavier, France). Cells were plated at a density of 2×10^6 cells/cm^2 in DMEM low glucose (DMEM-LG) (Lonza) containing 20% foetal bovine serum (FBS) (Lonza) and antibiotics. All cell cultures were performed at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h, MSC were selected by removing the non-adherent cells. Fourteen days later, adherent cells were detached with 0.25% trypsin-EDTA (Sigma) and plated at 2000 cells/cm^2. Medium was changed twice a week and cells re-plated when at 70% confluency.

4.2. *Isolation and culture of rNSC*

Cortex from E14–15 Sprague–Dawley rat embryos were dissected, cut into small pieces and finally triturated using a firepolished Pasteur pipette. Neurospheres were formed by culturing in 3v DMEM-HG, 1v Ham's F12 (Lonza) supplemented with B27 (GIBCO, Cergy-Pontoise, France), 20 ng/ml EGF, 20 ng/ml bFGF (R&D Systems Europe, Lille, France), 5 μg/ml heparin (Sigma) and antibiotics. Cells were fed every 2 or 3 days and split every 5 days at 1:3 dilution. Large neurospheres were cut with micro-scissors. Adherent neural progenitors were cultured with the same media in poly-D-lysine (Sigma) coated plates (100 μg/mL poly-D-lysine in Dulbecco's phosphate buffered saline, DPBS, Lonza).

4.3. *Synthesis of HEDP-coated SPIO nanoparticles*

SPIO nanoparticles were prepared and purified as previously described (Portet et al., 2001). Briefly, bare iron cores were generated under agitation by the coprecipitation of an aqueous solution of 100 μL Fe_2Cl_2 (0.314 M) and 100 μL Fe_3Cl_3 (0.666 M) in 2.5 mL of a 1 M tetramethylammonium hydroxide solution (all from Sigma). After 3 min, the 4 nm bare iron particles were incubated for 20 min at 20 °C with 48 μM 1-hydroxyethylidene-1.1-bisphosphonic acid (HEDP) (Sigma). The pH of the solution was lowered to 6.0 using 3 M tetraethylsulfamide (Sigma) to allow for HEDP adsorption on the bare cores. After incubation, the nanoparticles were purified on a 20 cm Biogel P6 column (BioRad, Marnes-la-Coquette, France) at room temperature (RT). This resulted in a solution of HEDP-coated SPIO nanoparticles with a hydrodynamic diameter of 20 nm and a final iron concentration of 0.8 g/L. Magnetic relaxivities of those nanoparticles at 7 T were: $r1=1$ mM^{-1} s^{-1} and $r2=50$ mM^{-1} s^{-1} (Mowat et al., 2007).

4.4. *Nanoparticle uptake and cell characterization*

4.4.1. *Iron labeling*

Nanoparticles were added to the culture medium at a concentration of 25, 50 or 100 μg iron/mL for 24 or 48 h after the rMSC reached 60% confluency. Fugene® (Roche, Meylan, France) transfection agent was tested to decrease the incubation time with nanoparticles: 1 μL/mL Fugene® was added to DMEM containing nanoparticles for 30 min before incubation with the cells in culture.

4.4.2. *Cell viability*

It was estimated by Trypan blue (Sigma) exclusion and by an MTS assay kit (Promega, Charbonnières-les-Bains, France). Briefly, after washing the cells with DPBS and incubation with diluted (1:5) MTS reagent for 4 h, samples were read at 490 nm by use of a Multiskan ascent spectrophotometer (Thermo Fisher scientific, Cergy Pontoise, France).

4.4.3. *Prussian blue (PB) staining*

Cells were fixed in 4% paraformaldehyde (PFA) (Sigma) for 15 min at 4 °C, washed with DPBS and incubated 20 min with Pearl's reagent. Reagent was extemporaneously prepared: 1% potassium ferrocyanide in 3% HCl (Sigma), heated for 20 min at 60 °C and filtered. Cells were counterstained with 1% eosin, washed with 1% acetic acid (Sigma) before dehydration and mounting (Eukitt) (Labonord, Templemars, France). Cells were examined under bright field using an Axioscop microscope (Carl Zeiss, Le Pecq, France), a CoolSnap ES camera (Photometrics, Tucson, Arizona) and Metavue analysis software (Roper Scientific, Evry, France).

4.4.4. *Quantitative intracellular iron content assessment*

The average iron content of rMSC was assessed after incubation using inductively coupled plasma optical emission spectroscopy. $5x10^5$ cells were incubated with 65% (v/v) nitric acid for

2 h. Cell extracts were diluted in water and the total iron content was measured at 260 nm using a Jobin-Yvon 238 ICP-OES spectroscope (Ultrace Instruments SA, Longjumeau, France).

4.4.5. TEM

Iron intracellular localization was confirmed by TEM. Cells were fixed for 30 min in 2% glutaraldehyde in DPBS, then washed with DPBS, post-fixed in 2% osmium tetroxide in DPBS for 1 h, and washed again before dehydration with ethanol (Sigma). After exposure to propylene oxide (Sigma), samples were infiltrated in 100% epon resin and polymerized in a 60 °C oven for 48 h. Samples were sectioned using a diamond knife on an Ultracut-S ultramicrotome (Leica Microsystems, Rueil-Malmaison, France). 70 nm sections were picked up on copper grids, contrasted with uranyl acetate (Merck, Fontenay Sous Bois, France) and Pb nitrate (Merck) solutions and coated with carbon. Sections were examined with a Jeol 2011 transmission electron microscope at 140 kV (Jeol, Croissy sur Seine, France).

4.4.6. SEM

Cells were fixed with 2% glutaraldehyde (Sigma), dehydrated with increasing concentrations of ethanol and desiccated with hexamethyldisilazane (Carl Roth, Lauterbourg, France). Samples were finally coated with carbon and examined with a Jeol 6301F scanning electron microscope at 11 kV for backscattered images. EDX analysis was performed to confirm the iron nature of the observed nanoparticles.

4.4.7. *In vitro* MRI

After incubation with the SPIO nanoparticles, cells were washed, trypsinized and centrifuged. Cells were then embedded in a low melting-point 0.5% agarose type VII (Sigma) gel at a concentration of 1 cell/voxel. MRI experiments were performed on a Bruker Avance DRX 300 system (Bruker Biospin, Wissembourg, France) equipped with a 150 mm vertical super-widebore magnet operating at 7 T, an 84 mm i.d. shielded gradient set capable of 144 mT/m maximum gradient strength and a standard 64 mm diameter birdcage resonator, operating on a Paravision software platform (version 2.1.1, Bruker Biospin). A 3D spoiled gradient-echo sequence (TR=110 ms; FOV 30*30*15 mm; matrix 128*128*32, α=20°), modified to a multi-echo sequence (TE=5 ms, IE=5 ms, nEchos=6) was used for quantitative T2* mapping (Franconi et al., 2006).

4.4.8. *Osteogenic differentiation*

rMSC were plated at a density of 5500 cells/cm^2 in DMEM-HG containing 10% FBS, 10 mM β-glycerol-phosphate, 50 mM ascorbate-2-phosphate, 100 nM dexamethason and antibiotics (Sigma). Medium was changed twice a week during 21 days. Cells were washed 3 times with DPBS and fixed with ice-cold 70° ethanol for 1 h at 4 °C. Staining was performed by 10 min incubation with 40 mM Alizarin Red-S (Sigma), pH 4.2 at RT. Cells were finally washed with DPBS and samples examined using an Axiovert 40 CFL bright-field microscope (Carl Zeiss).

4.4.9. *Neuronal differentiation*

Neuronal induction was performed according to a previously described protocol (Woodbury et al., 2000). Briefly, rMSC were plated at 10x10^3 cells/cm^2 on 0.5 µg/cm^2 fibronectin (Sigma) coated coverslips in DMEM-HG containing 20% FBS and antibiotics. Neuronal pre-induction medium, consisting of DMEM-HG containing 20% FBS and 1 mM β-mercaptoethanol (Sigma), was added for 24 h. Afterwards, cells were washed with DPBS and transferred to neuronal induction media composed of DMEM-HG, 2% dimethylsulfoxide (DMSO) (Sigma) and 200 µM butylated hydroxyanisole (Sigma). Uninduced cells and cells exposed to the induction medium for 24 h and 48 h were stained for nestin, β3-tubulin and NeuN to assess the extent of neuronal differentiation. After washing with DPBS, cells were fixed with 4% PFA at 4 °C for 15 min, permeabilized with 0.2% Triton X-100 (Sigma) for 5 min and blocked with DPBS, 10% normal goat serum (Sigma), 4% bovine serum albumin (BSA) (Sigma) at RT for 45 min. After washing with DPBS, the cells were then incubated overnight at 4 °C with 5 µg/mL anti-nestin (BD Biosciences, Le Pont De Claix, France), 3.8 µg IgG2b/mL anti-β3-Tubulin (Sigma) or 20 µg/mL anti-NeuN (Chemicon, St Quentin en Yvelines, France) monoclonal mouse antibodies in DPBS, 4% BSA, 0.2% Triton X-100. After washing with DPBS, incubation with 2.5 µg/mL secondary biotinylated anti-mouse antibody (Ref BA2001, Abcys, Paris, France) in DPBS, 4% BSA, 0.2% Triton X-100 was performed for 1 h at RT. Finally, after washing with DPBS, incubation with 20 µg/mL streptavidin FITC (Ref F0422, Dako, Trappes, France) in DPBS for 40 min at RT was made before mounting (Fluorescent mounting medium, Dako) and observation under fluorescence microscopy (Axioscop, Carl Zeiss). Isotype controls were performed.

4.5. *Labeling methods for histochemical detection*

4.5.1. *Hoechst 33342 labeling*

After washing, cells were resuspended in 1 ml DMEM-HG containing 10 mg/mL Hœchst (Sigma), left for 30 min at 37 °C and finally washed again 3 times.

4.5.2. *PKH26 labeling*

Cells were washed and resuspended in 500 µl DMEM-HG before adding 1.5 µL PKH26 (Sigma) for 3 min. Excess label was washed away with FBS, then washed again with DPBS 4 times. Co-cultures of GFP$^+$ cells and Hoechst or PKH26-labeled cells were performed for 24–72 h in order to evaluate the diffusion of the fluorescent dye to neighbouring cells. Cells were finally washed with DPBS and samples examined by fluorescence microscopy.

4.5.3. *Lentiviral transduction of GFP*

TEFLYGA (Gabon ape) human cell line containing a simian retrovirus encoding GFP with a CMV promoter was used for virus production (kind gift from Dr JC. Pagès). Infection was performed with 5 µg/ml Polybrene® (Sigma) and GFP$^+$ cells were selected with 500 µg/ml Neomycin G418 (Sigma) for 7 to 10 days.

4.5.4. *BrdU labeling*

rMSC and rNSC were incubated with 1 µL/mL BrdU from a BrdU Immunofluorescence Assay Kit (Roche Diagnostics, Meylan, France) on a time scale ranging from 6 to 96 h. For the rapidly dividing rNSC, shorter incubation times of 6 and 24 h were tested and the uptake efficiency was compared between

floating neurospheres and adherent neural progenitors. BrdU uptake was assessed by immunofluorescent staining following the Immunofluorescence Assay Kit instructions. After BrdU labeling, rMSC and rNSC were seeded on fibronectin and poly-D-lysine-coated coverslips respectively, washed with DPBS and fixed with 4% PFA in DPBS for 10 min at 4 °C. After washing, cells were incubated with the primary anti-BrdU mouse antibody (1:20) (Clone BMG 6H8) for 30 min. The secondary anti-mouse fluorescein-conjugated antibody (1:20) was added for 30 min at 37 °C. Mounting and observations were performed as described in the previous section.

4.6. *Cell transplantation*

A total number of 50 ten week old Sprague–Dawley female rats of 220 g were anesthetized with 16 mg/kg Xylasin (Rompun®, Bayer, Puteaux, France) and 80 mg/kg Ketamine (Clorketam 1000®, Vétoquinol, Lure, France). Prior to transplantation, rMSC were incubated for at least 72 h with BrdU and the SPIO nanoparticles were added to the medium during the last 48 h of incubation. rNSC (adherent neural progenitors) were incubated for 48 h with the SPIO nanoparticles and BrdU was added during the last 6 h of incubation. After labeling, 40×10^3 to 100×10^3 rMSC or rNSC were injected in a final volume of DMEM-HG ranging from 2 to 5 μL using a 10 μl microsyringe (Hamilton, Reno, Nevada). The injection was performed at a rate of 2 μL/min and the cannula was kept in situ for an additional 4 min before withdrawal. Control rats received an equivalent volume of DMEM-HG only or unlabeled cells. Injection coordinates were chosen in the SVZ, close to the beginning of the RMS: +0.9 mm rostral to Bregma, −1.2 mm lateral to the midline, −3.9 mm ventral to the dura and the tooth bar was set at −3.3 mm (Watson, 1986). For some rats, an OB lesion was performed just after grafting the cells, ipsilaterally to the injection site at 6.5 mm from Bregma, using a 3 mm diameter drill.

4.7. *In vivo immunohistochemical tracking*

Four, 14 and 21 days after transplantation, rats were anesthetized by CO_2 inhalation. 0.9% NaCl and 4% PFA were used sequentially for intracardial perfusion and brains were left in PFA containing increasing amounts of sucrose (from 2.5 to 20%) (all from Sigma). Brain sections of 14 μm were made on a CM 3050S cryotome (Leica Microsystems). IHC was used in vivo for the detection of $BrdU^+$ cells and a mouse anti-CD11b antibody (specific for macrophage-microglia) was used to assess the inflammatory reaction. For BrdU staining, sections were washed with DPBS and incubated for 2 h at 65 °C in SSC buffer (1v sodium chloride-sodium citrate pH 7.4, 1v formamide), 30 min with 2 N HCl in DPBS at 37 °C and finally 10 min in 0.1 M sodium borate, pH 8.5 at RT (all from Sigma). Quenching of peroxidases was made with 3% H_2O_2 (Sigma) in DPBS at RT for 15 min. For BrdU and CD11b staining, blocking was performed overnight at 4 °C or 40 min at RT, respectively. BrdU blocking buffer: DPBS, 3% BSA, 0.1% Tween 20 (Sigma). CD11b blocking buffer: DPBS, 10% normal goat serum, 4% BSA, 0.2% Triton-X 100. Sections were incubated with the rat anti-BrdU antibody (1:100) (Ref OBT0030, clone BU1/75 ICR1) for 4 h at RT and with the mouse anti-CD11b antibody (1:50) (Ref MCA275G, clone MRC OX-42) overnight at 4 °C (both from Abd Serotec, Cergy Saint-Christophe, France). After washing, sections were incubated at RT for 2 h with the secondary biotinylated anti-rat (1:200) (Ref BA4001) or for 1 h with the anti-mouse antibodies (1:200) (Ref BA2001). BrdU incubation buffer: DPBS, 1% BSA, 0.1% Tween 20. CD11b incubation buffer: DPBS, 4% BSA, 0.2% Triton-X 100. Incubation with Vectastain® ABC reagent in 0.1% Tween 20 in DPBS was made at RT (1.5 h for BrdU and 1 h for CD11b) (all from Abcys). Sections were washed and revealed with 0.03% H_2O_2, 2 mg/mL diaminobenzidine (DAB) (Sigma) (supplemented with 0.16% nickel chloride hexahydrate (Sigma) for BrdU), in DPBS for 10 min at RT and washed before mounting.

For PB/CD11b double staining, sections were fixed again with 2% PFA in DPBS for 5 min at 4 °C after CD11b staining. Staining with Pearl's reagent was then performed as previously described and samples were examined by bright field microscopy.

4.8. *In vivo MRI tracking*

In vivo, MRI was performed under the conditions defined for *in vitro* study. A TE=10 ms was chosen as it allowed to depict microscopic susceptibility inhomogeneities induced by SPIOs without jeopardizing the overall image quality by macroscopic susceptibility artefacts. During the *in vivo* imaging process, animals were anaesthetized using a mixture of isoflurane/O_2 (1.5%, 2 L/min) and body temperature was maintained at 36.5–37.5 °C using a feedback-regulated heating pad.

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CONCLUSION

Après avoir caractérisé *in vitro* l'absorption des nanoparticules de fer par les CSM, nous avons confirmé leur viabilité et la conservation de leur potentiel de différenciation en ostéoblastes ainsi qu'en cellules de phénotype neuronal après absorption. D'autres études ont déjà validé la différenciation des CSM vers d'autres phénotypes mésodermaux, par exemple en chondrocytes et adipocytes, après marquage par des nanoparticules de fer (Farrel, Wielopolski et al. 2008) et nous nous sommes donc contentés de valider la différenciation des CSM vers un seul phénotype mésodermal, le phénotype ostéocytaire. Il est important de noter que le protocole de différenciation neuronale utilisé dans cette étude fait désormais l'objet de controverses. En effet, ce protocole adapté de Woodbury et al. (Woodbury, Schwarz et al. 2000), est uniquement basé sur l'utilisation de dimethylsulfoxide (DMSO), de β-mercaptoethanol ainsi que de « butylated hydroxyanisole » (BHA). Ainsi, l'acquisition rapide de la morphologie neuronale observée au cours de ce protocole est parfois suspectée d'être due à un effondrement de l'architecture du cytosquelette en réponse à ces molécules chimiques (Neuhuber, Gallo et al. 2004; Bertani, Malatesta et al. 2005; Tao, Rao et al. 2005; Zurita, Bonilla et al. 2008). Cependant, même si un doute peut être émis à ce sujet, le point important de notre étude était la réponse identique des CSM à ce protocole de différenciation, qu'elles aient absorbé des nanoparticules ou non.

Les nanoparticules observables en IRM, ainsi que le marquage au BrdU, nous a permis de démontrer que les CSM restaient au niveau du site d'injection après transplantation et qu'elles ne migraient que si une lésion était présente au niveau du tissu cérébral, confirmant ainsi l'intégrité de leur potentiel de migration vers les lésions après marquage (Jendelova, Herynek et al. 2004; Sykova and Jendelova 2005; Yano, Kuroda et al. 2005). Une lésion, même à grande distance du site d'implantation au niveau du bulbe olfactif, provoquait en effet la migration des CSM de façon similaire aux CSN, qui sont connues pour leurs capacité de migration au sein de cerveaux adulte (Fricker, Carpenter et al. 1999; Englund, Fricker-Gates et al. 2002). De plus, le signal observé au niveau du bulbe olfactif provenait avec certitude des cellules transplantées puisque celui-ci n'était pas observable dans les cerveaux sans lésions (ni dans les cerveaux avec lésions, mais non-transplantés).

Cette étude est l'une des premières à décrire une migration de CSM sur une distance aussi importante (7 mm) mais ne fut finalement observée que par histologie, la lésion du bulbe olfactif créant de trop importants artefacts pour permettre une observation par IRM.

Ainsi, l'utilisation du marquage au bleu de Prusse et du BrdU ont permis une nette visualisation des cellules en migration, avec une faible fraction de ces cellules colocalisant avec la microglie (marquage OX42). De manière générale, seule une fraction des cellules implantées ont été détectées, et celles-ci étaient visibles en plus grand nombre 14 jours après implantation par rapport à 21 jours.

Pour conclure ce chapitre, la fraction de CSM survivantes après transplantation reste faible, confirmant ainsi la plupart des études de la littérature. Les CSM utilisées ne migrent pas significativement dans un contexte cérébral normal et leur différenciation en cellules de phénotype neuronal reste également très faible avec une légère expression de marqueurs neuraux (Nestine et β3-Tubuline) au niveau du site d'implantation 5 jours après la greffe. Cette étude confirme donc l'enjeu crucial que constitue l'amélioration de la survie ainsi que du potentiel de différenciation des CSM après transplantation, ce qui fait l'objet du troisième chapitre de ce travail de thèse.

CHAPITRE 3

Un pré-traitement en EGF-bFGF pour améliorer la différenciation neuronale de cellules stromales mésenchymateuses humaines

INTRODUCTION

Est-il possible d'améliorer simplement la différenciation neuronale de cellules stromales mésenchymateuses humaines avec un pré-traitement en facteurs de croissance lors de leur expansion ?

Ayant pour ultime objectif l'utilisation de CSM dans des études d'ingénierie tissulaire du cerveau, il semble important de pouvoir tout d'abord favoriser leur différenciation neuronale par un moyen simple. Plus particulièrement, la sous-population de CSM humaines MIAMI « marrow-isolated adult multilineage inducible » (D'Ippolito, Diabira et al. 2004) possède cette capacité de différenciation neuronale, un mécanisme dépendant du NT3, et semble donc particulièrement adaptée à cette application. Pour favoriser cette différenciation neuronale, il semble idéal de profiter du temps nécessaire à l'expansion des cellules *in vitro* avant implantation. Cette amélioration des capacités de différenciation pourrait permettre de favoriser l'intégration des cellules au sein du parenchyme cérébral et ainsi d'en augmenter la survie, maximisant de fait leurs effets possibles sur la réparation tissulaire.

Par conséquent, nous avons soumis les cellules MIAMI à l'effet combiné de deux facteurs de croissance : l'EGF et le bFGF. Ces facteurs de croissance sont principalement connus pour leurs effets mitogènes sur les cellules souches neurales (Temple 2001; Caldwell, Garcion et al. 2004; Tsai and Kim 2005), leur permettant également de conserver leur potentiel de différenciation en neurones et cellules gliales. De plus, certaines équipes ont montré que ces facteurs pouvaient induire l'expression de la nestine, un marqueur de CSN, dans des populations de CSM (Hermann, Gastl et al. 2004; Kim, Honmou et al. 2006; Choong, Mok et al. 2007; Song, Song et al. 2007; Yang, Mu et al. 2008). Ces études laissent donc supposer qu'il soit possible de favoriser l'induction d'un phénotype neuronal chez les CSM par l'utilisation d'EGF et de bFGF. Plusieurs études rapportent également l'utilisation de ces facteurs, par exemple pour traiter des CSM avant implantation dans des modèles de rats parkinsoniens (Levy, Bahat-Stroomza et al. 2008; McCoy, Martinez et al. 2008), mais la caractérisation détaillée des effets potentiels d'un tel pré-traitement en EGF-bFGF n'est que très peu-ou pas-documentée. Nous nous sommes donc attachés à comprendre les changements induits dans les CSM lors d'une exposition à ces facteurs en culture, avant de caractériser leur réponse au protocole de différenciation neuronale en 3 étapes, que nous avons précédemment décrit (Tatard, D'Ippolito et al. 2007).

Par comparaison à des CSM non-traitées, nous nous sommes donc tout d'abord intéressés aux bénéfices potentiels d'un traitement en EGF-bFGF pendant 10 jours en termes de spécification neuronale des cellules MIAMI. Nous avons particulièrement caractérisé la vitesse de prolifération des cellules en lien avec l'expression de marqueurs du cycle cellulaire, de marqueurs de cellules pluripotentes (*Nanog, Sox2* & *Oct4a*) ainsi que l'expression de marqueurs précoces impliqués dans l'acquisition d'un phénotype neural (*Notch1, HES5*, Nestin, *Pax6, Ngn2*). Ayant précédemment montré que l'acquisition de certaines caractéristiques neuronales par les cellules MIAMI dépendent de l'action du NT3, le potentiel de réponse à cette neurotrophine a également été évalué, avec et sans traitement en EGF-bFGF. Nous avons ainsi étudié la phosphorylation de la protéine Erk située en aval de la voie de signalisation du récepteur au NT3. Dans une seconde partie, nous avons caractérisé les effets de ce pré-traitement en EGF-bFGF sur la capacité des cellules MIAMI à se différencier vers un phénotype neuronal *in vitro*. Pour cela, des paramètres spécifiques d'une différenciation neuronale, comme la réduction de la prolifération, l'acquisition d'une morphologie neuronale ainsi que l'expression de marqueurs neuraux et neuronaux (β3-Tubuline et neurofilaments), ont été étudiés.

EGF and bFGF pre-treatment enhances neural specification and the response to neuronal commitment of MIAMI cells

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ABSTRACT

Background. Multipotent mesenchymal stromal cells raise great interest for cell therapy studies. Some MSC subpopulations have the potential to undergo neural differentiation, including MIAMI (Marrow Isolated Adult Multilineage Inducible) cells, which differentiate toward the neuro-ectodermal lineage in a multi-step neurotrophin 3-dependent manner. Epidermal and basic fibroblast growth factors are neural stem cell mitogens often used in neuronal differentiation protocols for MSCs with a limited understanding of their role. In this study, we have examined their capacity to enhance the neuronal differentiation of MIAMI cells.

Methods. We have characterized MIAMI cell neuronal differentiation program in terms of stem cell molecules, cell cycle control, the acquisition of a neuronal morphology and the expression of neural and neuronal molecules in the absence and presence of an EGF-bFGF pre-treatment

Results. EGF-bFGF pre-treatment down-regulated the expression of stemness markers *Oct4A, Notch1* and *Hes5,* whereas neural/neuronal molecules *Nestin, Pax6, Ngn2* and the neurotrophin 1 & 3 receptors were up-regulated. During differentiation, a sustained Erk phosphorylation in response to NT3 was observed, cells began to exit from the cell cycle, exhibited a decreased spreading of the cell body and increased neurite length. In addition, neuronal *β3-tubulin* and *Neurofilament* expression was increased, a preoligodendrocyte engagement was noted, and no default neurotransmitter phenotype was observed. Overall, mesodermal markers were unaffected or decreased, while neurogenic/adipogenic PPARγ2 was increased.

Discussion. EGF and bFGF pre-treatment enhances neural specification and the response to neuronal commitment of MIAMI cells which may be of benefit for applications in adult cell therapy of the nervous system.

Keywords: MSC, EGF, bFGF, neuronal development, cell therapy

INTRODUCTION

Stem cells hold a great promise in regenerative medicine due to their capacity to self-replicate and to produce various differentiated cell types. Nevertheless, the contribution of each stem cell type has to be clearly evaluated in the context of a specific pathology. Among the different sources of stem cells, adult multipotent mesenchymal stromal cells (MSCs) are interesting for cell therapy studies due to their large differentiation potential and their immunoregulatory properties (Le Blanc 2003; Le Blanc, Tammik et al. 2003; Gotherstrom, Ringden et al. 2004; Maitra, Szekely et al. 2004; Horwitz, Le Blanc et al. 2005; Nasef, Mathieu et al. 2007)**.** Moreover, their easy isolation from bone marrow and their self-renewal capacity may allow autologous grafts to be performed. A number of studies have shown that subpopulations of MSCs may differentiate into neural-like cells *in vitro*, either by genetic manipulation, with the help of co-culture systems or by using different inducer molecules and growth factors (Song and Sanchez-Ramos 2003; Hermann, Maisel et al. 2006; Ross and Verfaillie 2008). In regard of the difficulties in obtaining neural stem cells (NSCs) from adults and the inherent ethical problems linked with their isolation from foetal tissue, the identification of alternate sources of neuronal-like cells for cell therapy in the central nervous system is of great importance.

MSCs transplanted in adult rat brains may respond to microenvironmental cues and a fraction of them differentiate into neural-like cells (Kopen, Prockop et al. 1999; Zhao, Duan et al. 2002; Jendelova, Herynek et al. 2004). In addition, MSCs, that can migrate towards sources of lesions in the brain (Mahmood, Lu et al. 2002; Jendelova, Herynek et al. 2004; Hellmann, Panet et al. 2006; Sykova and Jendelova 2007a; Delcroix, Jacquart et al. 2009), may also provide a functional improvement in animal models, either directly or indirectly by their ability to produce various growth factors (Chen, Li et al. 2002; Li, Chen et al. 2002; Mahmood, Lu et al. 2002; Zhang, Li et al. 2005). Finally, their administration in the central nervous system is feasible and seems to be safe in human subjects (Bang, Lee et al. 2005). For all these reasons, MSCs may become a clinically viable technology for cell therapy of the central nervous system that is not hindered by ethical and tissue rejection-related concerns. However, further investigations are still required, particularly with human MSCs, to clearly evaluate their neuronal differentiation potential.

In the present study, we have used a subpopulation of developmentally immature human MSCs termed MIAMI (Marrow-Isolated Adult Multilineage Inducible) cells that express markers typical of embryonic stem cells and are capable of differentiating *in vitro* into

cell lineages derived from all three germ layers, as previously described (D'Ippolito, Diabira et al. 2004). Moreover, MIAMI cells, which are expanded under niche-like conditions, including a low oxygen tension environment, express the major receptor for hepatocyte growth factor, c-met (D'Ippolito, Diabira et al. 2004), as well as a number of chemokine and growth factor receptors, that may enhance their migratory potential leading to an improved regenerative potential (Rosova, Dao et al. 2008). We recently reported a 3-step neurotrophin 3 (NT3)-dependent neuronal differentiation of these cells using a protocol that aims to mimic the differentiation program of neural stem cells at the molecular level (Tatard, D'Ippolito et al. 2007). During *in vitro* neuronal differentiation, MIAMI cells progressively acquired a neuronal morphology and the expression of neural and neuronal molecules. At the end of the program, 50-60 % MIAMI cells became immature neuron-like cells with a smaller fraction showing ionic channel activity but no action potential. It is necessary to improve the neuronal differentiation of MIAMI cells, as well as to better understand their behavior, and the molecular mechanisms that direct it, in order to optimize their future use in cell therapy protocols.

Epidermal growth factor and basic fibroblast growth factor (EGF and bFGF) are powerful mitogens for NSCs and neural precursor cells from many different regions of the developing and adult central nervous system, as well as for ES cell-derived neural precursors (Reynolds, Tetzlaff et al. 1992; Guan, Chang et al. 2001; Temple 2001; Caldwell, Garcion et al. 2004; Tsai and Kim 2005). These factors allow the cells to retain their ability to further differentiate into neurons and glia. Moreover, bFGF may contribute to maintain the neurogenic niche *in vivo* (Mudo, Bonomo et al. 2009). Interestingly, these factors may also permit the long-term proliferation of human mesencephalic precursors when expanded under hypoxia (Schwarz 2007). It has been shown that MSCs or subpopulations of MSCs may already express certain neural-related genes (Woodbury, Reynolds et al. 2002; Tondreau, Lagneaux et al. 2004), and it was therefore assumed that an EGF-bFGF exposure of MIAMI cells may further improve their *in vitro* neuronal differentiation. Indeed, in MSCs, the expression of Nestin, a marker specific for NSCs (Gilyarov 2008), was up-regulated after EGF and bFGF exposure under adherent (Song, Song et al. 2007) or non-adherent conditions prior to *in vitro* neurogenesis (Hermann, Gastl et al. 2004; Kim, Honmou et al. 2006; Yang, Mu et al. 2008). Furthermore, recent studies described the differentiation of MSCs, after a first-step treatment with EGF-bFGF, into dopamine producing cells leading to a functional improvement in a rat model of Parkinson's disease (Barzilay, Kan et al. 2008; Levy, Bahat-Stroomza et al. 2008). However, a precise characterization of the impact of an EGF-bFGF

pre-treatment, prior to MSC neuronal differentiation, by comparing to a control and extensively analyzing several neuronal molecules is still lacking (Hermann, Gastl et al. 2004; Long, Olszewski et al. 2005; Kim, Honmou et al. 2006; Song, Song et al. 2007; Yang, Mu et al. 2008). It is therefore interesting to assess the benefits of using EGF-bFGF in more detail prior to the induction of a neuronal-like differentiation of MIAMI cells.

In this study, we sought to first assess the potential benefits in terms of neuronal lineage specification of an EGF-bFGF pre-treatment during the expansion of MIAMI cells, isolated from two different anatomical sites, to further improve their neuronal differentiation *in vitro*. The effects of the EGF-bFGF pre-treatment was characterized and compared on MIAMI cells harvested from vertebral bodies or from iliac crest of living donors. As NT3 is essential for the MIAMI cells neuronal-like differentiation, we also studied the effect of EGF-bFGF on the response of MIAMI cells to NT3 by evaluating the expression of the NT3 receptors and Erk phosphorylation involved in this transduction pathway. A neuronal differentiation program involves a reduction of cell proliferation leading to post-mitotic cells, a process accompanied with a loss of stem cell molecules together with the acquisition of a neuronal morphology and the expression of neural and neuronal molecules. Therefore, we further analysed the MIAMI cells, with or w/o pre-treatment, all along the differentiation protocol in terms of acquisition of a neuronal morphology, reduction of proliferation, apoptosis and molecular expression pattern.

MATERIALS AND METHODS

Bone marrow harvesting, selection & expansion of MIAMI cells. Whole bone marrow was obtained from vertebral bodies (T1–L5) of a 3 year old male cadaveric donor who died of fatal traumatic injury (MIAMI #519) (D'Ippolito, Diabira et al. 2004) or from iliac crest of a 20 year old male living donor (Lonza Walkersville, Maryland; MIAMI #3515), following guidelines for informed consent set by the University of Miami School of Medicine Committee on the Use of Human Subjects in Research. As previously described (D'Ippolito, Diabira et al. 2004), isolated whole bone marrow cells were plated at a constant density of 10^5 cells/cm^2 in DMEM-low glucose media, containing 5 % fetal bovine serum (FBS) (Hyclone, South Logan, Utah) and antibiotics (AB) (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B) (Sigma, St Quentin Fallavier, France) on a fibronectin (Sigma) substrate. Whole bone marrow cells, containing adherent and non-adherent cells, were incubated at 37°C under hypoxic conditions (3 % O_2, 5 % CO_2 and 92 % N_2). Seven days later, half of the culture medium was replaced. Fourteen days after the initial plating, the non-adherent cells were removed. Pooled colonies of adherent cells were carefully rinsed and plated at low density for expansion (100 cells/cm²) on 1.25 ng/cm² fibronectin. Cells were expanded in DMEM-low glucose (Gibco, Cergy Pontoise, France), 3 % FBS and AB (40 mL/175 cm² flask) under hypoxic conditions. Cells were fed every 2-3 days by changing half the medium and split every 5 days, keeping 1/4 of old medium. Presented results were obtained with vertebral bodies-isolated MIAMI cells, unless otherwise stated.

Neural stem cells. For use as positive controls, foetal neuroepithelial progenitor cells were kindly provided by Dr. Micheline McCarthy (Dept. of Neurology, Univ. of Miami, Miller School of Medicine) and cultured as previously described (Tatard, D'Ippolito et al. 2007).

EGF-bFGF pre-treatment. A 10 days EGF & bFGF pre-treatment was performed using either a low dose (5 ng/mL) or a high dose (20-50 ng/mL) of both EGF and bFGF (R&D Systems Europe, Lille, France) supplemented with 5 µg/mL heparin (Sigma) and a lipid mixture (working concentration of 510 nM lipoic, 70 nM linolenic and 150 nM linoleic acid, all from Sigma). Unless otherwise stated, presented results were mainly obtained with 20 ng/mL of EGF-bFGF. To investigate the NT3 response of pre-treated cells, we checked the neurotrophin receptor expression in response to 0, 50 and a higher dose (100 ng/mL) of EGF-bFGF after pre-treatment. As we already observed an effect at the intermediary dose (50 ng/mL), we continued with this dose for the detection of Erk/pErk. Briefly, cells were treated

with NT3 (30 ng/mL) for 5, 10 and 15 minutes following a serum deprivation phase of 24 hours prior processing for western blotting.

MIAMI cells neuronal-like differentiation. This 3-step differentiation process was described in greater detail elsewhere (Tatard, D'Ippolito et al. 2007). Briefly, MIAMI cells passages 5 to 9 were seeded at 3000–4000 cells/cm^2 on a 2 µg/cm^2 fibronectin-coated surface in DMEM-F12 (GIBCO) medium supplemented with 20 % FBS, 10 ng/mL bFGF, antibiotics and cultured for 24 h (Neural specification, step 1). At the end of the neural specification treatment, cells were washed and neuronal commitment (step 2) was induced by exposing the cells to 30 ng/ml NT3 (R&D Systems) in the presence of 1 mM β-mercaptoethanol (Sigma), for 2 days. Neuronal differentiation (step 3) was induced by rinsing and then exposing the cells to 100 µM butylated hydroxyanisole, 25 mM KCl, 2 mM valproic acid, 4 µM forskolin, 1 µM hydrocortisone, 5 µM insulin, 5 mM Hepes, 10 µM rolipram (all from Sigma), 30 ng/mL NT3, 10 ng/mL NGF (R&D Systems), and 30 ng/mL BDNF (R&D Systems) for 3 days. When the EGF-bFGF pre-treatment was used before neuronal differentiation, the specification medium (step 1) was replaced by DMEM-F12 containing 20 % FBS, 20 ng/mL of both EGF-bFGF and 5 µg/mL heparin. Use of insulin-transferrin-selenium and lipid media supplement (ITS+3, Sigma) was also tested during the differentiation protocol (working concentrations of 10 µg/ml insulin, 5.5 µg/ml apotransferrin, 5 ng/ml sodium selenite, 4.7 ng/ml linoleic acid, 4.7 ng/ml oleic acid and 0.5 µg/ml BSA). Neuronal differentiation was performed at ambient atmospheric oxygen pressure (21 % pO_2).

Intracellular labeling & flow cytometry. MIAMI cells were washed with DMEM-low glucose and detached with 10 mL Versene (Lonza) for 20 min at 37°C. After pelleting, cells were washed with DPBS and fixation was performed in 0.25 % formaldehyde (Sigma) for 30-60 minutes at 4°C. After fixation, cells were pelleted, permeabilized with 0.5 % Tween 20 (Sigma) in DPBS for 15 min at 37°C and then rinsed with DPBS, 2 % FBS, 0.02 % azide, 0.2 % Tween 20. Incubation with mouse anti-Nestin antibody (1:15, clone 3k1, #ab6320, Abcam, Paris, France) or IgG1k isotypic antibody (#557273, BD Biosciences, Le Pont De Claix, France) in DPBS, 2 % FBS, 0.02 % azide was made for 1 h at 4°C. After washing, FITC-conjugated anti-mouse antibody (1:50, #F0479, Dako, Trappes, France) was added for 30 min at 4°C. The cells were washed and finally fixed in DPBS, azide, 0.7 % formaldehyde. Fluorescence signals were acquired with a FACScalibur flow cytometer (BD Biosciences) and data analysed with the Cellquest software (BD Biosciences). The background level was estimated using the fluorescence signal of the isotypic control. Human NSCs were used as a positive control.

Primer design & validation. Human sequences were determined using Pubmed nucleotide search (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) websites. The online freeware Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used for primer modelling, clustalw (www.ebi.ac.uk) to align nucleotidic sequences and nucleotide blast (www.ncbi.nlm.nih.gov) to confirm the specificity of the defined primer sequences. When possible, pairs of primers were designed across intron-spanning regions to avoid genomic DNA contamination. Sense and antisense desalted primer pairs (Eurogentec, Angers, France or Origen, USA) were mixed in RNAse free water at a final concentration of 5 µM and validated using cDNA from expanding or differentiated MIAMI cells, expanding or differentiated foetal neuroepithelial cells and commercial qPCR Human Reference cDNA (Clontech, Takara bio, Saint-Germain-en-Laye, France). The melting peak of the amplicon had to be narrow and unique, and its size and specificity was confirmed by electrophoresis. Finally, a serial dilution of the PCR product was re-amplified to draw a linear curve Ct=f (Quantity). The efficiency of the primer was calculated from the slope of the linear curve: $E=[10^{(-1/slope)}-1]x100$. Only primer pairs with an efficiency greater than 80 % were validated for use (table 4).

Gene	Full name	NM accession number	Sequences	Amplicon
AChE	*Acetylcholinesterase*	015831-00665	F=5'-CCCTCTCGAAACTACACG-3' R=5'-GGTCCAGACTAACGTACTGC-3'	163
β3-Tubulin	*Homo sapiens tubulin, beta 3*	006086	F=5'-CCAGTATGAGGGAGATCG-3' R=5'-CACGTACTTGTGAGAAGAGG-3'	185
CCND1	*Cyclin D1*	053056	F=5'-CTCTCCAAAATGCCAGAG-3' R=5'-GATGGACAGGAAGTTGTTGG-3'	186
ChAT	*Choline acetyltransferase*	020549-020984-020985-020986	HS_CHAT_1_SG QUANTITECT PRIMER ASSAY, REF QT00029624, QIAGEN	Unknown
DAT	*Dopamine transporter*	001044	F=5'-GAAGGTGGTATGGATCACAG-3' R=5'-GTAGAAGTCAACGCTCAGGT-3'	121
EGFR	*EGF receptor*	005228	F=5'-CAGCCACCCATATGTACC-3' R=5'-TGGAGTCTGTAGGACTTGG-3'	184
FGFR1	*FGF receptor 1*	015850-023105-023106-023110-023111	F=5'-GGTAACTCTATCGGACTCTCC-3' R=5'-GTGGAAGTCACTCTTCTTGG-3'	198
GAD 25/67	*Glutamate decarboxylase*	000817-013445	F=5'-CCTGGAAGAGAAGAGTCG-3' R=5'-CTCCTCACCGTTCTTAGC-3'	166
GalC	*Galactosylceramidase*	000153-001037525	F=5'-GTTGCCTTATGGGAGATG-3' R=5'-AAGCCATCAGTCAGAGGCTAC-3'	183
GFAP	*Glial fibrillary acidic protein*	002055	F=5'-TTGAGAGGGACAATCTGG-3' R=5'-CGACTCAATCTTCCTCTCC-3'	161
*HES5**	*Hairy and enhancer of split 5*	001010926	F=5'-AGCCCCAAAGAGAAAAACCGA-3' R=5'-GCTGTGCTTCAGGTAGCTGAC-3'	183
*LpL**	*Lipoprotein lipase*	000237	F=5'-ACAAGAGAGAACCAGACTCCAA-3' R=5'-AGGGTAGTTAAACTCCTCCTCC-3'	149
MAP1b	*Microtubule-associated protein 1b*	005909	F=5'-CCTCGAGACGTGATGAGTGA-3' R=5'-TTGGGCGTCAGAGAGAAGTT-3'	437
Msi1	*Homo sapiens musashi homolog 1*	002442	F=5'-AGGTGAAGGAGTGTCTGG-3' R=5'-TTCTTCGTTCGAGTCACC-3'	196
Nanog	*Nanog homeobox*	024865	F=5'-TCTCCAACATCCTGAACC-3' R=5'-AGTAGAGGCTGGGGTAGG-3'	153
Nes	*Nestin*	006617	F=5'-AGAAACAGGGCCTACAGAG-3' R=5'-AAAGCTGAGGGAAGTCTTG-3'	170
*NFL**	*Neurofilament, light polypeptide*	006158	F=5'-ATGAGTTCCTTCAGCTACGAGC-3' R=5'-GGGCATCAACGATCCAGAGC-3'	198
NFM	*Neurofilament, medium polypeptide*	005382	F=5'-GACCTCAGCAGCTACCAG-3' R=5'-CTAGTCTCTTCACCCTCCAG-3'	170

*NFH**	*Neurofilament, heavy polypeptide*	021076	F=5'-GCAGTCCGAGGAGTGGTTC-3' R=5'-TAGCGTCTGTGTTCACCTTGG-3'	71
*Ngn2**	*Neurogenin 2*	024019	F=5'-CGCATCAAGAAGACCCGTAG-3' R=5'-GTGAGTGCCCAGATGTAGTTGTG-3'	173
Notch1	*Notch homolog 1, translocation-associated*	017617	F=5'-GACCTCATCAACTCACACG-3' R=5'-AGAAACAGGGGTGTCTCC-3'	198
*Nurr1 (NR4A2)**	*nuclear receptor subfamily 4, group A, member 2*	006186	F=5'-TTGCCAGATGCGCTTCGACG-3' R=5'-CCAACAGCCAGGCACTTCTG-3'	414
Oct4A	*Pou class 5 homeobox 1*	002701	F=5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3' R=5'-GGCAGATGGTCGTTTGGCTGAATA-3'	186
*Olig2**	*0ligodendrocyte lineage transcription factor 2*	005806	F=5'-GCTGCGACGACTATCTTCCC-3' R=5'-GCCTCCTAGCTTGTCCCCA-3'	244
*Pax6**	*Paired box 6*	000280-001604-001127612	F=5'-AGGTATTACGAGACTGGCTCC-3' R=5'-TCCCGCTTATACTGGGCTATTT-3'	104
p21	*Cyclin-dependent kinase inhibitor 1A*	000389-078467	F=5'-GCAGAGGAAGACCATGTG-3' R=5'-CCTCTTGGAGAAGATCAGC-3'	171
PPAR gamma	*Peroxisome proliferator-activated receptor gamma*	138712-015869-138711-005037	F=5'-AACAGATCCAGTGGTTGC-3' R=5'-CTCCACAGACACGACATTC-3'	176
Rex1	*Zinc finger protein 42 homolog*	174900	F=5'-CTG AGT ACA TGA CAG GCA AG-3' R=5'-CTC AAC TTC CTA GTG CAT CC-3'	169
*Runx2**	*Runt-related transcription factor 2*	001024630-004348	F=5'-TCCTATGACCAGTCTTACCCCT-3' R=5'-GGCTCTTCTTACTGAGAGTGGAA-3'	190
Sox2	*Sex determining region Y-box 2*	003106	F=5'-GCAGTACAACTCCATGACC-3' R=5'-AGGAAGAGGTAACCACAGG-3'	161
*Sox9**	*Sex determining region Y-box 9*	000346	F=5'-GCCAGGTGCTCAAAGGCTA-3' R=5'-TCTCGTTCAGAAGTCTCCAGAG-3'	213
Sp7 (Osterix)*	*Sp7 transcription factor*	152860	F=5'-CCCAGGCAACACTCCTACTC-3' R=5'-GGCTGGATTAAGGGGAGCAAA-3'	175
SSEA4 synthase	*ST3 beta-galactoside alpha-2,3-sialyltransferase 3*	174963-174964-174965	F=5'-TGAAGATGGGACTCTTGG-3' R=5'-CACTGAATCTGCAACAGG-3'	176
*TH**	*Tyrosine hydroxylase*	199292-000360-199293	F=5'-CACCATCTAGAGACCCGGCC-3' R=5'-GCAATCAGCTTCCTGCGCTG-3'	290

Table 4. Human specific primer sequences

*Primers ordered from Origen, USA and runs performed in Miami's facilities.

RT-qPCR. During expansion and at the various steps of the differentiation protocol, MIAMI cells were detached using trypsin-EDTA (Sigma) and washed in DPBS. Following the manufacturer's guidelines, cells were lysed in a 1 % β-mercaptoethanol containing buffer and RNA extracted following a treatment by DNAse to remove any traces of genomic DNA (Total RNA isolation Nucleospin® RNA II, Macherey Nagel, Hoerdt, France). First strand cDNA synthesis was performed with a Ready-To-Go You-Prime First-Strand Beads® kit in combination with random hexamers (Amersham Biosciences, Orsay, France) using 1 µg RNA according to the manufacturer's guidelines. Following first strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France), eluted in 50 µL RNAse free water (Gibco). Five microliters of cDNA (1:20) were mixed with iQ SYBR Green Supermix (Biorad) and primer mix (0.2 µM) in a final volume of 15 µL. Amplification was carried on a Chromo4 thermocycler (Biorad) with a first denaturation step at 95°C for 3 min and 40 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 15 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. A mean cycle threshold value (Ct) was obtained from 2 measurements for each cDNA. Several candidate genes were tested for normalization [Glyceraldehyde-3-phosphate

dehydrogenase (Gapdh, NM_002046), Hypoxanthine phosphoribosyltransferase 1 (Hprt1, NM_000194), Beta actin (Actb, NM_001101), 30S ribosomal protein S18 (Rps18, NM_001093779), Heat shock 90 kD protein 1 beta (Hspcb, NM_007355), Eukaryotic translational elongation factor 1 alpha (Ef1a, NM_001402), Ribosomal protein L13a (RpL13a, NM_01242), Ubiquitin C (UbC, NM_021009) & Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide variant 1 & 2 (YWHAZ, NM_003406 & NM_145690)] and we used the GeNorm™ freeware (www.primerdesign.co.uk/geNorm.asp) to determine that Gapdh, Hprt1 & Hspcb were the three most stable housekeeping genes. Normalization was performed with at least two housekeeping genes (Ef1a/RpL13a) in Miami's facility. The relative transcript quantity (Q) was determined by the delta cT method $Q=E^{(\text{Ct min in all the samples tested-Ct of the sample})}$ 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. (Vandesompele, De Preter et al. 2002). Q normalized = Q/(geometric mean of the 3 most stable housekeeping genes Q). All samples were run in duplicate. On figures, results are expressed as a percentage of change in the expression of the mRNA relative to the expression in expanding MIAMI cells ± average deviation (% change mRNA X= $[(Q_x-Q_{\text{expanding MIAMI cells}})/Q_{\text{expanding MIAMI cells}}]$ x 100). The experimental details outline was performed following the guidelines of the SCCAN core facility ("Service Commun de Cytométrie et d'Analyse Nucléotidique", Angers, France).

Western blotting. Cell protein extracts were separated into Triton X-100 soluble and insoluble lysates. Protease inhibitor cocktail (Sigma) was added to both lysis buffers directly prior to use. The cell pellet was resuspended in buffer A [500 mM Tris HCl pH 6.8, 50 mM EGTA, 1 M KCl, 10 % Triton X-100 (all from Sigma)], triturated, vortexed and centrifugated at 13000 g for 20 minutes to pellet the Triton X-100 insoluble pellet. Buffer A containing the supernatant Triton X-100 soluble fraction was stored at -80°C. The Triton X-100 insoluble pellet was washed twice with buffer B [500 mM TrisHCl pH6.8, 50 mM EGTA, 850 mM sucrose, 10 % Triton X-100 (all from Sigma)] then centrifugated at 13000 g for 20 minutes. Buffer B was aspirated from the Triton X-100 insoluble pellet and the pellet was reconstituted in Laemmli buffer [10 % SDS, 500 mM Tris HCl pH 6.8, 4 % glycerol, 1 % β-mercaptoethanol, bromophenol blue (all from Sigma)], boiled for 5 minutes and stored at -80°C. The protein concentration of the Triton X-100 pellet was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Primary antibodies included rabbit anti-NTrK1 (1:2000, polyclonal, #sc-118, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NTrK3 (1:500, clone 75213, #MAB3731, R&D Systems), mouse anti-pErk p44/42 (1:2000,

#9106S, Cell Signaling Technology, Danvers, MA, USA), mouse anti-Erk p44/42 (1:2000, #9107, Cell Signaling Technology), goat anti-pTau (Ser396) (1:500, polyclonal, #sc-12414, Santa Cruz Biotechnology) & mouse anti-Tau (1:500, monoclonal, #sc-32274, Santa Cruz Biotechnology). Secondary antibodies used were sheep anti-mouse IgG-horseradish peroxidase (HRP) (1:2000, GE Healthcare, USA), goat anti-rabbit IgG-HRP and bovine anti-goat IgG-HRP (1:1000, both from Santa Cruz Biotechnology) depending on the primary antibody. 50 µg protein extracts were electrophoresed on 6 % or 10 % gels and transferred onto Immobilon-P 0.2 µM membranes (Millipore). Membranes were blocked with 5 % BSA-DPBS for 1 hour then incubated with primary antibody in 5 % BSA-DPBS. Secondary antibodies were diluted in DPBS alone and incubated on the membrane for 45 minutes. Membranes were developed with ECL Plus Western Blot Detection System (GE Healthcare) and an AFP Imaging Mini-Medical Services Auto developer. Image analysis was completed using ImageJ (http://rsbweb.nih.gov/ij/).

Caspase 3 assay. MIAMI cells were plated in 1.9 cm² fibronectin-coated wells and Caspase 3 activity was assessed following the Caspase 3 assay kit (Sigma) guidelines. Briefly, positive controls were induced by exposing the cells to Staurosporine (1 µg/mL, Sigma) for 4 hours at 37°C. After apoptotic induction of the positive controls, cell culture medium of every well was replaced by assay buffer and frozen at -80°C. After thawing, Caspase 3 substrate (Ac-DEVD-AMC) was added and the solutions transferred to black plates (Greiner bio-one, Courtaboeuf, France). Fluorescence was read in a kinetic mode for 1 hour at room temperature using a Fluoroskan Ascent FL microplate reader (Thermo Fisher scientific, Illkirch, France) with excitation/emission wavelengths of 355 nm-460 nm, respectively. Caspase 3 activity was read from a 7-amino-4-methylcoumarin standard curve. Caspase 3 specificity of the fluorescent signal was assessed with the Caspase 3 inhibitor (Ac-DEVD-CHO). Every condition was performed in triplicate.

Immunocytofluorescence (ICF). Uninduced and induced cells at the end of step 2 (neuronal commitment) and step 3 (neuronal differentiation) of neuronal induction were used for Nestin, β3-Tubulin and NFM ICF. After washing the slides 3 times with DPBS, cells were fixed with 4 % paraformaldehyde at 4°C for 15 min and then permeabilized with 0.2 % Triton X-100 (Sigma) for 5 min. Slides were blocked with DPBS, 10 % normal goat serum (Sigma), 4 % bovine serum albumin (BSA) (Sigma) at room temperature for 45 min. After washing, slides were incubated overnight at 4°C with anti-NFM (1:50, clone NN18, #N5264, Sigma), anti-β3-Tubulin (1:1000, clone SDL.3D10, #T8660, Sigma) or anti-nestin (1:200, clone 10C2, #MAB5326, Chemicon, St Quentin en Yvelines, France) antibodies in DPBS, 4 % BSA,

0.2 % Triton X-100. Isotypic controls were made with IgG1k (clone MOPC-31C, #557273, BD Biosciences) and IgG2bk (clone 27-35, #555740, BD Biosciences). After rinsing, the cells were incubated with the secondary biotinylated anti-mouse antibody (1:200, Abcys) in DPBS, 4 % BSA, 0.2 % Triton X-100 for 1 hour. Finally, after rinsing again and following incubation with streptavidin-FITC (1:50, #F0422, Dako) in DPBS for 40 min, the slides were mounted (Dako) and observed with a fluorescence microscope (Axioscop, Carl Zeiss, Le Pecq, France), a CoolSnap ES camera (Photometrics, Tucson, Arizona) and Metavue software (Roper Scientific, Evry, France).

Scanning electron microscopy (SEM). Cells were cultivated on glass slides, fixed with 2 % glutaraldehyde (Sigma) and post-fixed with 2 % osmium tetroxide. Cells were then dehydrated with increasing concentrations of ethanol (Sigma) and transferred into acetone (Sigma) prior drying in a Bal-Tec CPD 030 critical point dryer (Bal-Tec, Liechtenstein). Samples were finally coated with carbon and examined with a Jeol 6301F scanning electron microscope (Jeol, Croissy sur Seine, France) at 11 kV for backscattered images.

Oil red staining. Oil red o (Sigma) solution (0.36 %) was made in 60 % isopropanol (Sigma) and agitated overnight before filtration. Medium was removed and cells fixed with 4 % paraformaldehyde during 30 to 40 min. Cells were washed with DPBS and water before staining with oil red solution for 50 min. Samples were finally washed with water and observed with a bright field microscope (Axiovert 40 CFL, Carl Zeiss).

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 a Student's t-test modified for small samples with $t'_0=(m_1-m_2)/\sqrt{(s_1^2/n_1+s_2^2/n_2)}$. Differences were considered significant if $|t'_0|> t_{k':0.05}$, with k' being the closer integer of the calculated $k=(s_1^2/n_1+s_2^2/n_2)^2/[(1/(n_1-1)(\ s_1^2/n_1)^2)+(1/(n_2-1)(s_2^2/n_2)^2)]$. Kruskal-Wallis test was used for multiple comparisons. Threshold P-value was set to 0.05.

RESULTS

I. EFFECTS OF EGF-bFGF ON MIAMI CELLS

EGF and bFGF receptors, *EGFR* and *FGFR1* respectively, were strongly detected by RT-qPCR, indicating that MIAMI cells may respond to these stimuli. In the following section, we first focus on the effects of an EGF-bFGF exposure on MIAMI cells during expansion.

Expression of stem and early neural-related genes during EGF-bFGF pre-treatment

RT-qPCR demonstrated that expanding, non pre-treated or EGF-bFGF pre-treated MIAMI cells expressed the stemness makers *Rex1* and *SSEA4 synthase*, as well as *Sox2* and *Nanog* at low levels. The stemness marker *Oct4A,* present at higher level, was down-regulated after treatment with EGF-bFGF for 10 days (2.5 ± 0.49 fold decrease).

MIAMI cells expressed a panel of early neural-related genes, including *Nestin, MAP1b, Msi1, Notch1, HES5, Pax6* & *Ngn2*. *Nestin* and *MAP1b* were the most strongly expressed and EGF-bFGF pre-treatment altered the expression of most of these genes. Indeed, the expression of the NSC marker *Nestin* was stimulated (2.9 ± 0.79 fold increase) and this was confirmed by flow cytometry and ICF with 5 and 20 ng/mL EGF-bFGF (figure 7A, B). Importantly, a higher concentration did not further increase *Nestin* expression. Moreover, Nestin was detected in almost all the non pre-treated or pre-treated cells but its expression always remained lower than for neural stem cells, used as a positive control (data not shown). *Pax6* increased after 20 ng/mL pre-treatment (1.8 ± 0.08 fold increase) and about 4 fold at 50 ng/mL; whereas *Notch1* expression, involved in NSC proliferation, slightly decreased (1.4 ± 0.05 fold decrease). Expression of *HES5* decreased and became almost undetectable while *Ngn2* increased (2.1 ± 0.08 fold), probably due to *Notch1* down-regulation. *Galactosylceramidase* (*GalC*), specifically expressed in oligodendrocytes, was also slightly up-regulated upon pre-treatment (1.8 ± 0.27 fold increase). Importantly, expression of stem and early neural-related genes was similar for iliac crest-derived MIAMI cells isolated from an older individual; with equivalent changes induced in response to EGF-bFGF exposure. Oligodendrocyte lineage transcription factor 2 (*Olig2*), another early marker of oligodendrocytes, was never detected in MIAMI cells.

All these results illustrate an EGF-bFGF induced specification of the MIAMI cells towards a neural phenotype, independently from their anatomical site of origin.

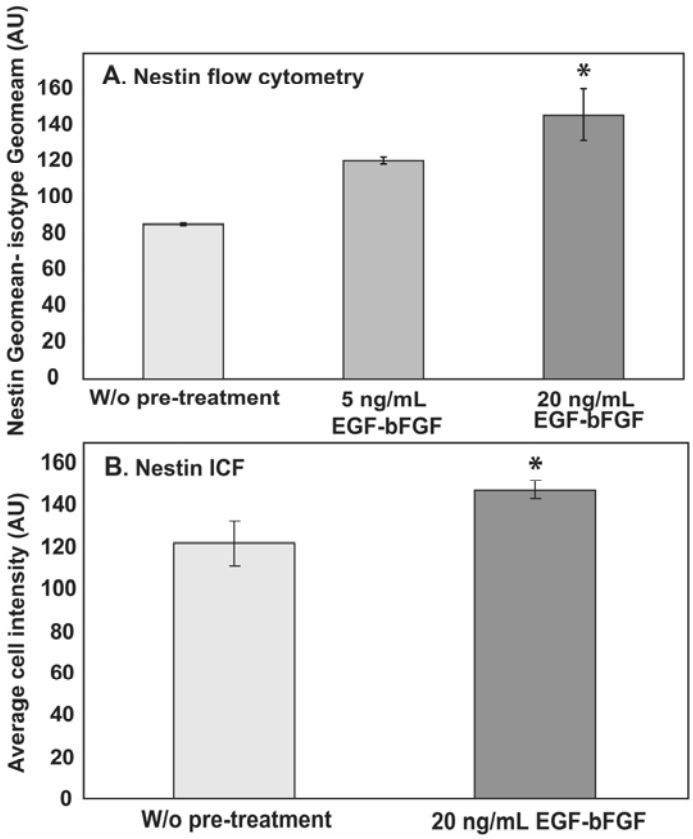


Figure 7. Nestin up-regulation during EGF-bFGF pre-treatment

Nestin expression by MIAMI cells increased after exposure to EGF-bFGF. More than 99 % pre-treated or non pre-treated MIAMI cells were nestin-positive. After pre-treatment, expression by flow cytometry increased (A) and was confirmed by ICF (B). Depicted results were obtained from a representative experiment and differences were considered significant at P=0.05*.

Effect of EGF-bFGF on the NT3 transduction pathway

Analysis of the NT3 receptor, neurotrophin tyrosine receptor kinase 3 (NTrK3), found that both the full length isoform (gp145, FL-NTrK3) and the tyrosine kinase deficient isoform (gp100, Tkd-NTrK3) of NTrK3 were up-regulated along with NTrK1, after EGF-bFGF pre-treatment (figure 8A). However, EGF-bFGF pre-treatment did not further up-regulate NTrK3 expression in iliac-crest isolated MIAMI cells, which express relatively higher basal levels of this receptor. In these cells, NT3 stimulation (30 ng/mL) induced Erk1/2 phosphorylation (p42/p44), which peaked after 5 min and dramatically decreased after 10 min of stimulation in the absence of EGF-bFGF pre-treatment. In contrast, EGF-bFGF pre-treatment resulted in a more sustained phosphorylation of pErk,1/2 both p42 and p44, lasting more than 10 min after NT-3 stimulation compared to non pre-treated cells (figure 8B, C). This result suggests a differential response to NT3 stimulation after EGF-bFGF pre-treatment.

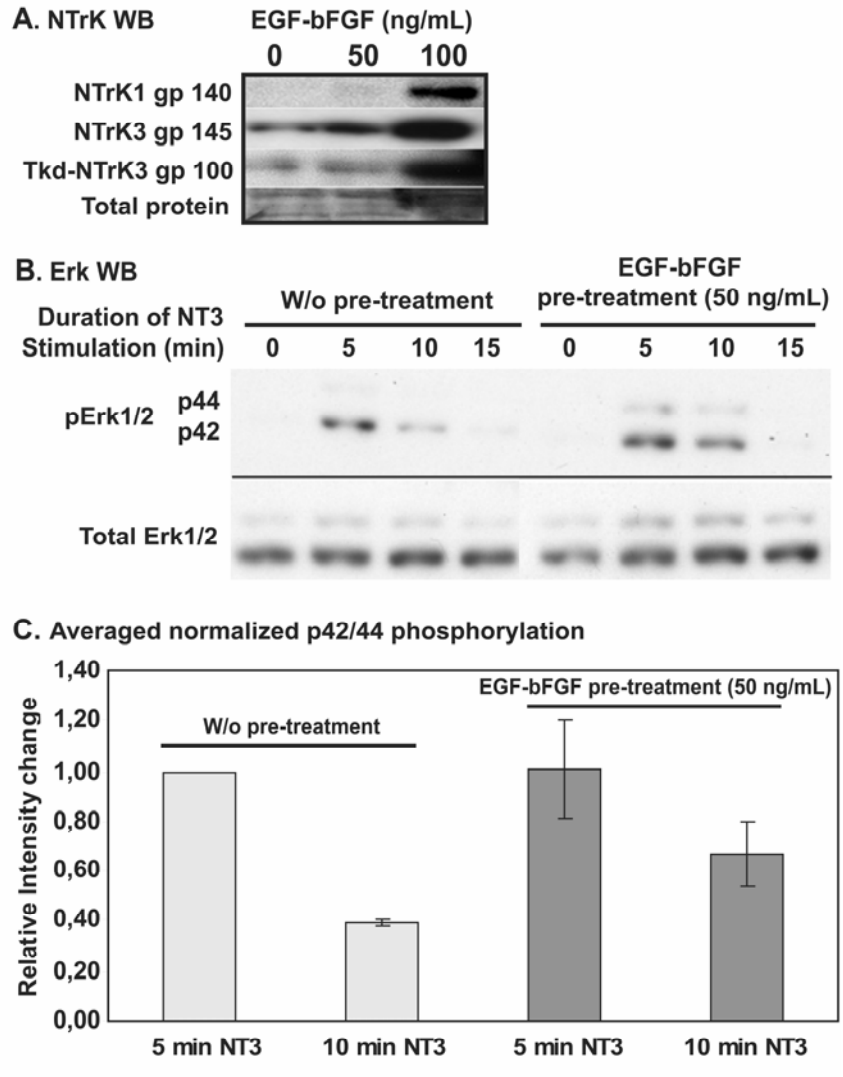


Figure 8. Representative NTrK up-regulation and Erk sustained phosphorylation

During EGF-bFGF pre-treatment, expression by MIAMI cells of NTrK1, NTrK3 and Tkd-NTrK3 receptors were strongly up-regulated (A). In response to NT3 stimulation, Erk phosphorylation peaked after 5 minutes and decreased at 10 min for non pre-treated cells. After 50 ng/mL EGF-bFGF pre-treatment, pErk peaked at 10 minutes but remained at a higher level after 10 minutes of stimulation; this sustained phosphorylation was similar for p42 and p44 (B, C). Abbreviations: NTrK: neurotrophic tyrosine kinase receptor.

Cell proliferation

The antiproliferative gene *p21* was up-regulated at the end of the pre-treatment (10.2 ± 0.74 fold increase). Trypan blue cell counting showed that the proliferation rate of EGF-bFGF pre-treated cells was slightly lower than for non pre-treated cells (3.2 *vs* 2.9 population doublings in 5 days). Moreover, no dead cells were detected, therefore confirming the increased p21 expression observed. The major changes induced by the EGF-bFGF pre-treatment in expansion are summarized in table 6, page 113.

II. EFFECTS OF THE EGF-bFGF PRE-TREATMENT ON NEURONAL DIFFERENTIATION

In this second section, we describe the results of EGF-bFGF pre-treatment on the neuronal differentiation of MIAMI cells to better understand the effects of this pre-treatment.

Decreased proliferation during neuronal differentiation

During neuronal differentiation, Trypan blue counting confirmed that non pre-treated cells continued to proliferate whereas cell proliferation was decreased in cells treated with 5 ng/mL EGF-bFGF and was almost abolished in 20 ng/mL EGF-bFGF pre-treated cells (figure 9A). Importantly, it should be noted that there were almost no dead cells detected.

Notch1 expression, which was already down-regulated at the end of the pre-treatment, further decreased during neuronal differentiation, both for non pre-treated and EGF-bFGF pre-treated cells (figure 9B). Moreover, expression of the cell cycle markers *p21* & *Cyclin D1* at the RNA level confirmed the decreased proliferation observed during differentiation after pre-treatment (figure 9C, D). Indeed, *p21* was low in non pre-treated cells, it increased during differentiation in 5 ng/mL pre-treated cells and it was already elevated after 20 ng/mL pre-treatment, prior to the beginning of the differentiation. Conversely, *Cyclin D1* expression was high in non pre-treated cells and increased during differentiation. In 5 ng/mL EGF-bFGF pre-treated cells, it decreased during differentiation whereas it was always low in cells pre-treated with a higher dose of EGF-bFGF.

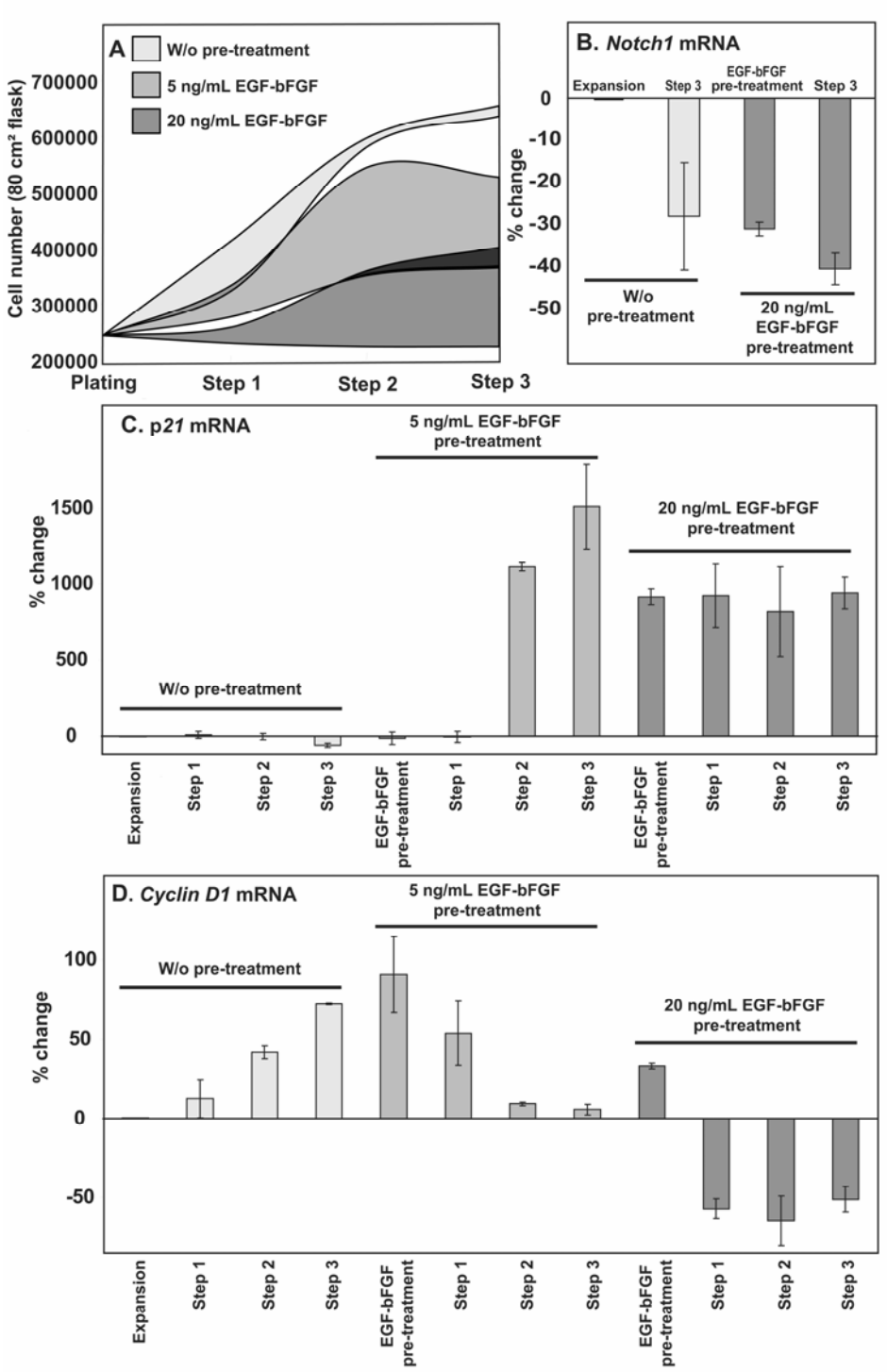


Figure 9. Reduced cell proliferation and cell cycle exit during neuronal differentiation

Trypan blue exclusion (A) demonstrated that the number of cells increased during differentiation while almost no increase was detected with 20 ng/mL EGF-bFGF pre-treated MIAMI cells. Analysis of *Notch1* (B) and cell cycle genes (*p21* & *Cyclin D1*, C & D respectively) expressions confirmed the reduced cell proliferation and cell cycle exit, observed during differentiation, after pre-treatment.

To confirm that there was no contribution from apoptosis to the reduced proliferation rate observed for pre-treated cells during neuronal differentiation, a Caspase-3 apoptosis assay was performed at the end of the differentiation protocol. No Caspase-3 activity was detected strongly suggesting that MIAMI cells, pre-treated or not with EGF-bFGF, did not undergo detectable apoptosis. In addition, it is interesting to note that at the end of differentiation, non pre-treated and EGF-bFGF pre-treated cells were sensitive to an apoptotic stress induced by 1 µg/mL staurosporine (figure 10A). When exposed to staurosporine, the cells exhibited an increased Caspase 3 activity as well as a retracted morphology and nucleus fragmentation (figure 10B, C). However, under expansion conditions, both non pre-treated and pre-treated MIAMI cells seemed to be resistant to the proapoptotic inducer staurosporine as Caspase-3 activity was never detected (figure 10A).

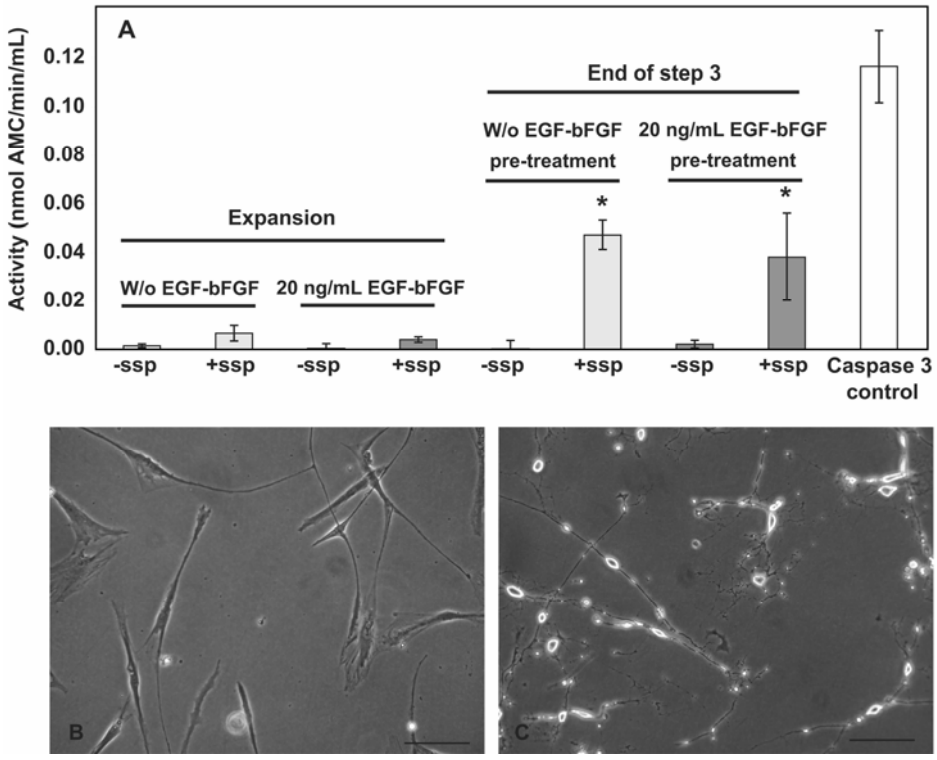


Figure 10. Caspase 3 activity in expansion and at the end of neuronal differentiation

Non pre-treated and EGF-bFGF pre-treated cells during expansion and after differentiation (step 3) did not show any Caspase 3 activity (A). A significant increase in Caspase 3 activity was detected only in differentiated cells treated with 1 µg/mL staurosporine whereas proliferative cells were resistant to staurosporine treatment. The top right bar of the histogram was obtained with a caspase 3 solution as a positive control. Picture C depicts morphology of pre-treated MIAMI cells induced by an apoptotic stimulus after differentiation compared to non apoptotic pre-treated MIAMI cells (B). Abbreviations: Ssp: staurosporine. *Significantly different medians at P=0.05. Scale bars: 100 µm.

Homogeneous cell morphology and induced neurite extensions

Cell density was a critical parameter for differentiating MIAMI cells toward a neuronal-like phenotype. Cells were expanded under low density (plating at 100 cells/cm²) and 3000 cells/cm² was optimal for plating prior to neuronal induction. Morphology was greatly impaired by a higher cell density, with decreased neurite formation. On the other hand, a lower plating density prior to neuronal induction resulted in the death of a large fraction of cells during differentiation.

Under low density expansion conditions, MIAMI cells treated with a combination of EGF-bFGF exhibited a more homogeneous morphology with a refractile body (figure 11A, B). Cell morphology is more heterogeneous when confluency was sufficiently high to allow cell contact. This effect was minimized when cells were pre-treated with EGF-bFGF. In addition, EGF-bFGF pre-treatment resulted in important morphological changes reminiscent of neurons at the end of the differentiation protocol (step 3). EGF-bFGF pre-treated cells were thinner and exhibited longer neurites compared to non pre-treated cells (figure 11C, D & table 5). Pre-treated cell neurites exhibited numerous connexions observed by SEM imaging at the end of differentiation (figure 11E, F). Similar effects were observed on iliac crest-isolated MIAMI cells.

	Non pre-treated cells	**EGF-bFGF pre-treated cells**
Cell area (µm²)	4378 ± 665	2893 ± 499*
Cell length (µm ± SD)	289 ± 43	421 ± 50*

Table 5. Cell area and length at the end of differentiation

EGF-bFGF pre-treated cells exhibited a decreased cell area corresponding to a narrower cell body and an increased cell length, due to the presence of long neurites at the end of step 3. Data represent the average length of the 3 longer cells of 5 different images in a representative experiment. * Significantly different means at P=0.05.

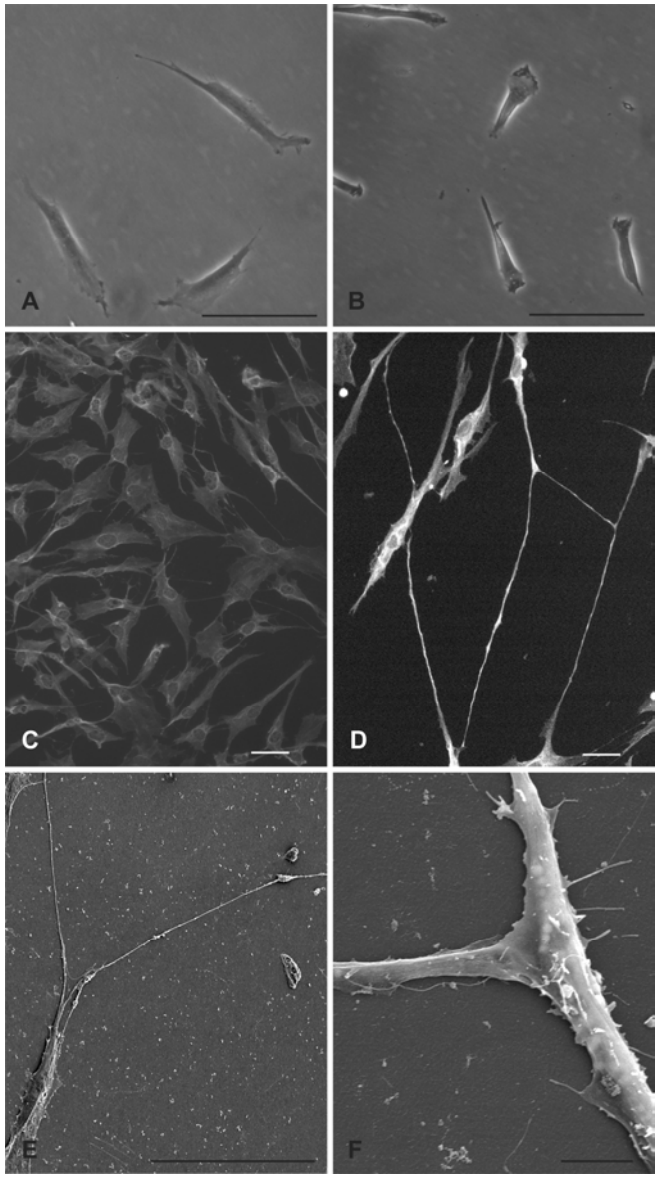


Figure 11. Induced neural/neuronal morphology by EGF-bFGF pre-treatment

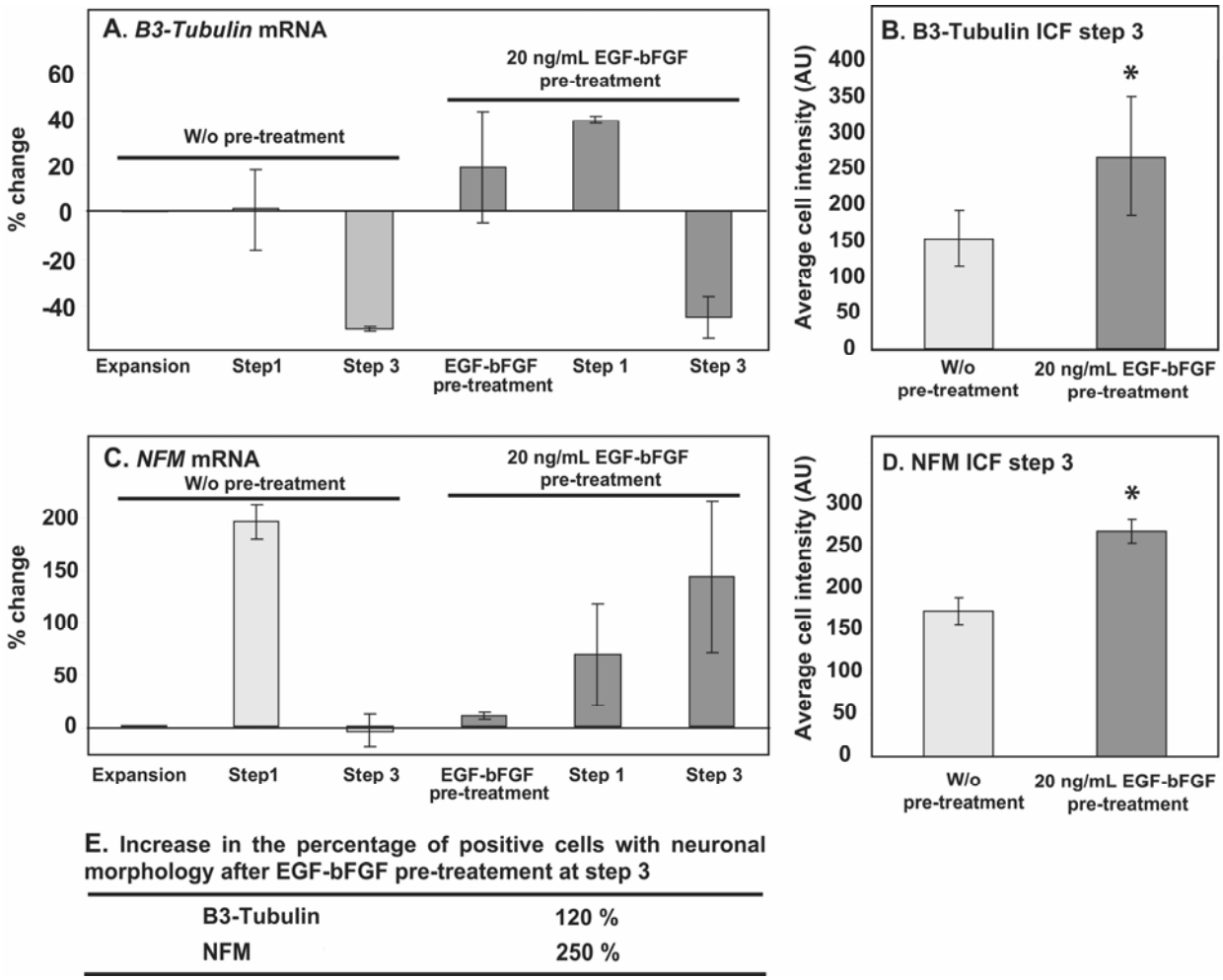
Under low density expansion conditions, non pre-treated MIAMI cells (A) were larger than pre-treated cells which were narrower and refractile (B). At the end of differentiation, EGF-bFGF pre-treated cells exhibited longer neurites as observed after NFM ICF (D) compared to non pre-treated cells (C). Neurites and interconnexions of EGF-bFGF pre-treated cells are depicted on SEM images at the end of differentiation (E, F, respectively). Scale bars A-E: 100 µm; F: 5 µm.

Improved neuronal expression pattern during neuronal differentiation

Nestin expression was up-regulated during pre-treatment and its expression decreased during differentiation as observed by ICF at the end of step 3 and even more drastically after 12 days of step 3 compared to expanding cells. No significant differences were observed with or w/o pre-treatment (data not shown). Noteworthy, *Nestin* mRNA remained high throughout the *in vitro* neuronal differentiation.

The pattern of expression of the early neuronal marker *β3-Tubulin* followed the neuronal developmental program only for EGF-bFGF pre-treated cells during the differentiation protocol. β3-Tubulin mRNA increased during neuronal differentiation before decreasing at the end of step 3 (figure 12A). Moreover, its expression was already high after pre-treatment compared to non pre-treated cells. Using ICF, β3-Tubulin expression at the end of step 2 and 3 was always significantly higher for EGF-bFGF pre-treated cells compared to non pre-treated cells, both in terms of positive cell percentage and marker intensity (figure 12B, E). In addition, assessment of β3-Tubulin expression at the end of step 2 by ICF confirmed that removal of NT3 lowered the percentage of β3-Tubulin positive cells at the end of step 2 (data not shown) as previously described (Tatard, D'Ippolito et al. 2007), further supporting the role of NT3 on the neuronal differentiation of MIAMI cells.

The mRNA expression of the mature neuronal marker *NFM* was strongly increased after step 1 for non pre-treated cells and further underwent a strong decrease at the end of step 3. In contrast, expression of *NFM* increased throughout the differentiation protocol for EGF-bFGF pre-treated cells (figure 12C). NFM protein expression was higher for pre-treated cells at the end of step 3 (figure 12D, E). *NFL, NFM* and *NFH mRNA* were all up-regulated at the end of step 2 when iliac crest-isolated MIAMI cells were pre-treated with EGF-bFGF compared to non pre-treated cells (54.29 ± 9.25, 6.85 ± 0.20 & 1.97 ± 0.26 fold increase for *NFL, NFM* and *NFH*, respectively). Moreover, the unphosphorylated microtubule associated protein Tau, characteristic of immature neurons, was readily detectable throughout the neuronal differentiation procedure by Western blot while we did not detect its phosphorylated form (data not shown).



E. Increase in the percentage of positive cells with neuronal morphology after EGF-bFGF pre-treatement at step 3

B3-Tubulin	120 %
NFM	250 %

Figure 12. Improved β3-Tubulin and NFM expression pattern during neuronal differentiation

EGF-bFGF pre-treated cells exhibited an increase and a final decrease *of β3-Tubulin* mRNA expression during differentiation (A), as well as a constant increase in *NFM* mRNA (C), similarly to what is observed during neuronal development *in vivo*. At the protein level, β3-Tubulin and NFM expression were higher for pre-treated cells as observed by ICF in terms of marker intensity (B, D respectively). EGF-bFGF pre-treatment also resulted in an increased number of for B3-Tubulin and NFM-positive cells with a neuronal morphology (E). Presented ICF data were obtained from a representative experiment and differences were considered significant at P=0.05*.

Neuronal neurotransmitter phenotypes during neuronal differentiation

Acetylcholinesterase (*AChE*), expressed by cholinergic and noradrenergic neurons, was slightly detected and increased during neuronal differentiation, especially for 20 ng/mL pre-treated MIAMI cells. Glutamate decarboxylase, (*GAD 25/67*), specifically expressed by GABAergic neurons and choline acetyltransferase (ChAT), another cholinergic phenotype enzyme were never detected. Dopaminergic phenotype mRNAs for tyrosine hydroxylase, dopamine transporter (*TH* & *DAT* respectively) and *Nurr1* were not detected or at very low levels.

Glial phenotypes during neuronal differentiation

Galactosylceramidase (*GalC*), characteristic of oligodendrocytes, was slightly expressed by MIAMI cells and further increased during neuronal differentiation for pre-treated cells, whereas its expression does not significantly vary in non pre-treated cells (figure 13A). Olig2 was not detected during differentiation. *Glial fibrillary acidic protein* (*GFAP*), specific for astrocytes, was never detected. Previous results suggested that an insulin, transferin, selenium and lipids complement (ITS+3) improved cell morphology and membrane potential when used in step 3 (data not shown). However, when cultured in this lipid rich medium all along the differentiation protocol, numerous perinuclear lipid vesicles appeared at the end of step 2 and remained until the end of step 3, in a more significant manner for pre-treated cells, as observed with oil red staining (figure 13C, D). The lipidic nature of these vesicles was confirmed using back-scattered electron SEM imaging (figure 13B), with visualization of osmium on double bonds-molecules rich sites. Nevertheless, exposure to ITS+3 during neuronal differentiation never modified the neurogenic/adipogenic *peroxisome proliferator-activated receptor gamma* (*PPAR gamma)* expression and the adipogenic *lipoprotein lipase* (*LpL)* remained undetected.

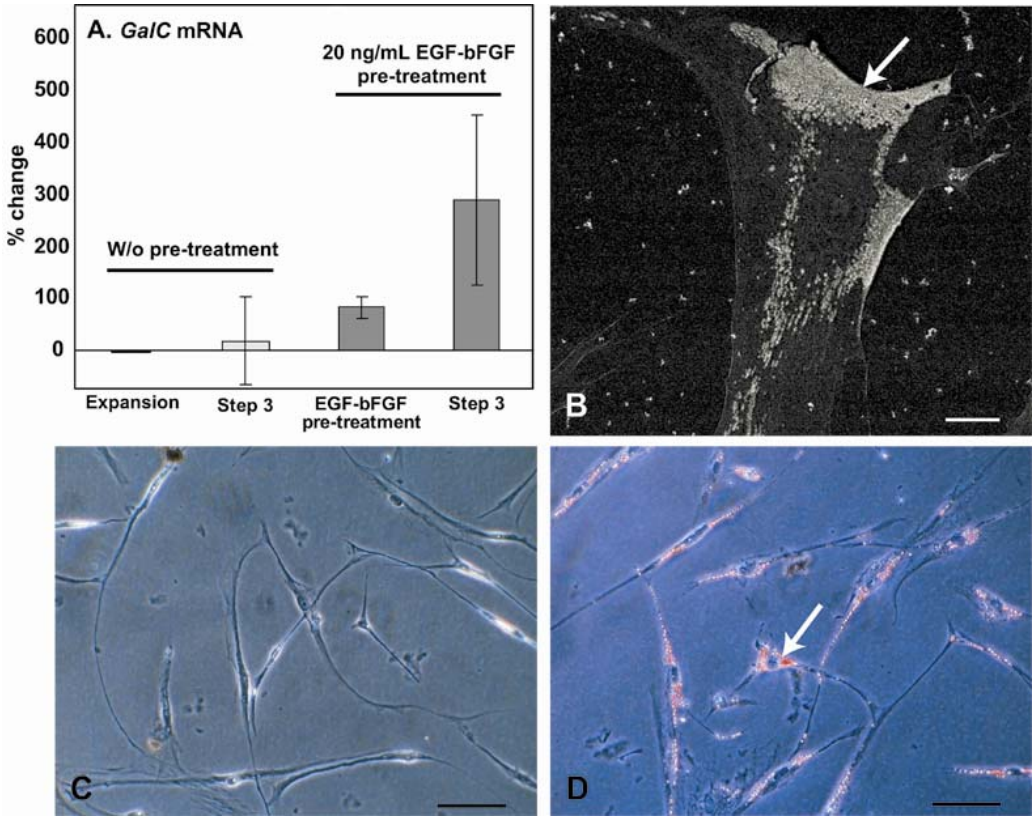


Figure 13. MIAMI cell potential to differentiate toward an oligodendrocyte lineage

After EGF-bFGF pre-treatment, GalC expression increased during differentiation of MIAMI cells (A). Use of ITS+3 during the neuronal differentiation protocol led to the formation of lipid vesicles (B, D, white arrowheads), especially in EGF-bFGF pre-treated cells. In presence of ITS+3, MIAMI cells exhibited red vesicles (D) at the end of differentiation compared to control cells (C) as observed with oil red staining. The lipidic nature of the vesicles observed in EGF-bFGF pre-treated cells was confirmed by SEM. Osmium specifically links to double bounds-molecules such as lipids and back-scattering imaging reveals the localization of the osmium inside the cells (B). Scale bars B: 20 µm ; C, D: 100 µm.

Mesodermal phenotypes during neuronal differentiation

We analysed the expression of several genes related to mesodermal phenotypes, the major differentiation pathways of MSCs. *Sox9*, specific to chondrogenic progenitors, was slightly up-regulated during neuronal differentiation of non pre-treated cells (1.47 ± 0.09 fold increase); whereas it was down-regulated when cells were pre-treated with EGF-bFGF (2.47 ± 0.56 fold decrease). The osteo-chondro marker *Runt-related transcription factor 2* (*Runx2*) was detected at basal levels and did not change during neuronal differentiation, while the osteogenic marker *Osterix* (*Sp7*) was never detected. *PPAR gamma*, commonly related to the adipogenic/neurogenic lineage, was expressed by MIAMI cells. It was further up-regulated

during differentiation, without significant changes with or w/o pre-treatment (11.4 ± 4.0 fold increase) whereas the solely adipogenic *LpL* was never detected.

The major changes observed during neuronal differentiation, with and without EGF-bFGF pre-treatment are summarized in table 6.

In expansion		**During neuronal differentiation**	
Transcription factors		**Receptors**	
Sox2	<>	*Notch1*	↓
Nanog	<>	**Cytoskeletal molecules**	
Oct4a	↓	*B3 Tubulin*	↑ then ↓
HES5	↓	*NFL*	↑
Pax6	↑	*NFM*	↑
Ngn2	↑	*NFH*	↑
Cytoskeletal molecules		**Cell cycle**	
Nestin	↑	*p21*	<>
Cell cycle		*Cyclin D1*	↓
p21	↑	**Glial molecule**	
CyclinD1	<>	*GalC*	↑
Other		*GFAP*	<>
Msi1	<>	**Proliferation rate**	
Receptors		↓	
Notch1	↓		
NTrK1	↑		
NTrK3	↑	**Legend**	
Tkd-NTrK3	↑	Unchanged	<>
Proliferation rate		Decreased	↓
↓		Increased	↑

Table 6. Major effects of EGF-bFGF pre-treatment

DISCUSSION

The differentiation of MSC subpopulations into neuronal-like cells, a tightly controlled process involving a series of stimuli, has been recently reported with different induction protocols (Ross and Verfaillie 2008). In order to be able to use these cells in future cell therapy studies, it is important to have a deeper understanding of the molecular events mediating this process. In a previous study, we reported that human MIAMI cells can be induced to differentiate, in a NT-3 dependent manner, to cells with morphological, biochemical and electrophysiological characteristics of immature neurons, therefore representing an autologous source of cells for the repair of damaged neuronal tissue. We here demonstrate that an EGF-bFGF pre-treatment of MIAMI cells initiated their cell cycle exit, directed their gene expression pattern towards a neural/neuronal lineage, enabling them to better respond to the differentiation stimuli. NTrK1 and NTrK3 receptors, both mediators of NT3 responses (Reichardt 2006; Ivanisevic, Zheng et al. 2007), were up-regulated, which was accompanied by a more sustained phosphorylation of Erk in response to NT3. During the induction protocol, the pre-treated MIAMI-derived neuronal-like cells stopped proliferating, presented longer neurites and acquired an expression pattern more consistent with a neuronal differentiation program. These results shed light to the molecular events induced by an EGF-bFGF pre-treatment during the neuronal differentiation of MSC subpopulations. Thus, this pre-treatment may increase the benefit of using MSC subpopulations in cell therapy approaches of the nervous system.

MIAMI cells have a broad differentiation potential, express the embryonic master transcription factors Rex1, Sox2, Nanog and Oct4, essential for the pluripotency and self-renewal properties of stem cells (Boyer, Lee et al. 2005; Masui, Nakatake et al. 2007). Some of these factors, as well as SSEA-4 synthase, have already been detected in previous studies (D'Ippolito, Diabira et al. 2004; Tatard, D'Ippolito et al. 2007). The variant A of Oct4, playing a major role in maintaining the stemness of cells (Liedtke, Stephan et al. 2008), was down-regulated by the EGF-bFGF pre-treatment, suggesting a certain commitment of the cells towards a differentiation program as previously suggested (Hermann, Gastl et al. 2004). Interestingly, a recent study demonstrates that Oct4 is required and sufficient to directly reprogram NSCs to pluripotency (Kim, Sebastiano et al. 2009). Nestin expression by pluripotent stem cells is considered to be a prerequisite for the commitment of the cells toward the neural lineage (Wislet-Gendebien, Leprince et al. 2003; Shiota, Heike et al. 2007) and this intermediate filament protein is commonly used to identify NSCs and neural

precursors (Gilyarov 2008). Corroborating the neural engagement of the MIAMI cells, Nestin expression was observed by flow cytometry in virtually all the MIAMI cells, as previously described for some MSCs; although results vary in terms of the percentage of positive cells depending on the detection method, cell species and expansion method used (Wislet-Gendebien, Leprince et al. 2003; Hermann, Gastl et al. 2004; Tondreau, Lagneaux et al. 2004; Song, Song et al. 2007; Tatard, D'Ippolito et al. 2007). Most importantly, EGF-bFGF pre-treatment induced Nestin over-expression and this confirmed previous RT-PCR and ICF studies performed on adherent (Long, Olszewski et al. 2005; Shiota, Heike et al. 2007) and non-adherent MSCs (Hermann, Gastl et al. 2004). Interestingly, a recent study describes, in a different cell type, a similar Nestin induction in response to EGF, a mechanism that may be mediated through the Erk dependent pathway (Huang, Shi et al. 2009).

Notch1, which is generally involved in maintaining an undifferentiated state and whose levels of expression are directly correlated to the proliferation of NSCs (Kageyama, Ohtsuka et al. 2005), was down-regulated in response to EGF-bFGF pre-treatment. In addition to Nestin over-expression and consistent with Notch1 down-regulation, Ngn2, an early neuronal marker, was over-expressed after EGF-bFGF pre-treatment, a response that have previously been described in Nestin expressing NSCs-derived neurons after treatment with bFGF (Vergano-Vera, Mendez-Gomez et al. 2009). The decrease of Notch1, which positively regulates HES5 transcription (Kageyama, Ohtsuka et al. 2005), could have led to a further decreased expression of HES5 after EGF-bFGF pre-treatment. Pax6, known to promote neurogenesis in neural stem cells (Kallur, Gisler et al. 2008) was also upregulated after pre-treatment, suggesting a potential progression from a neural to a more neuronal phenotype.

Cell cycle gene analysis and cell counting confirmed the propensity of EGF-bFGF pre-treated cells to decrease their proliferation rate and exit the cell cycle in expansion and during neuronal differentiation, in opposition to non pre-treated cells that continued to proliferate. During differentiation, non pre-treated cells exhibited a high level of Cyclin D1, the key cell-cycle regulatory protein governing progression from the G1 to S phase, and low level of the antiproliferative gene p21, a Cdk and PCNA inhibitor (Luo, Hurwitz et al. 1995). This cell cycle gene expression pattern is specific for integrin-mediated adhesion to fibronectin (Danen and Yamada 2001), the substrate used in this study. Conversely, pre-treatment slightly decreased the proliferation rate of MIAMI cells and up-regulated p21 during the expansion phase. Moreover, it further abolished cell proliferation during neuronal differentiation, maintained the levels of p21 and resulted in a down-regulation of Cyclin D1. These events,

that take place during the exit from the cell cycle, have already been described in the context of terminal differentiation (Ekholm and Reed 2000; Pacary, Legros et al. 2006). In addition to its anti-proliferative role, p21 may rescue human MSCs from apoptosis induced by low density culture (van den Bos, Silverstetter et al. 1998), with p21 loss increasing apoptosis (Seoane, Le et al. 2002). In our study, we did not observe apoptotic cells, neither in low density expansion nor in differentiation. However, differentiated MIAMI cells became sensitive to the pro-apoptotic molecule used as a positive control, confirming a previous report with MSCs (Pelletier, Oliver et al. 2005). We may hypothesize that inhibition of Caspase 3, or of another related molecule, occurs in expansion and that this inhibition is abolished during differentiation. For example, the heat shock protein Hsp20, highly expressed by MIAMI cells (data not shown), has recently been described to inhibit Caspase 3 via an interaction with the proapoptotic protein Bax (Fan, Chu et al. 2005; Fan, Ren et al. 2005).

Altogether, these results demonstrated that EGF-bFGF pre-treatment started to commit MIAMI cells toward the neural/neuronal lineage with a strong propensity to exit from the cell cycle during differentiation, which suggests cells are on their way to ultimately become post-mitotic. Moreover, the neuronal gene expression pattern closely mimicked the neuronal developmental program. *In vivo*, Nestin is down-regulated during the switch from neural precursors to differentiated post-mitotic neurons (Zimmerman, Parr et al. 1994; Gilyarov 2008). In this study, with or without pre-treatment, we still observed a high level of Nestin mRNA at the end of the differentiation protocol as described by others (Hermann, Gastl et al. 2004; Shiota, Heike et al. 2007), but its expression decreased at the protein level, confirming our previously reported results (Tatard, D'Ippolito et al. 2007). This led us to assume that Nestin expression may be regulated at the post-translational level as already suggested (Lei, Yongda et al. 2007). During the neuronal differentiation of pre-treated MIAMI cells, β3-Tubulin expression pattern was similar to that of neuronal precursors, with a first increase followed by a terminal decrease at the end of the neuronal differentiation protocol (Ginzburg, Teichman et al. 1985). NFL, NFM and NFH expression increased during neuronal differentiation, therefore following the expression pattern observed during normal brain development (Moskowitz and Oblinger 1995), whereas NFM decreased strongly at the end of step 3 for non pre-treated cells. In support of these results, we observed by ICF that compared to non-pre-treated cells, both NFM and to a certain extent β3-Tubulin were more strongly expressed at the end of step 3 in pre-treated cells that were narrower and exhibited longer neurites consistent with a neuronal morphology. We observed by ICF that only a subpopulation of narrow cells responded, while large and flat cells did not. In addition, a

higher percentage of NFM and β3-Tubulin-positive cells were observed at the end of the neuronal induction when the MIAMI cells were pre-treated. Therefore, EGF-bFGF pre-treatment enhanced the neuronal differentiation of MIAMI cells, although a mature phenotype was not observed as the neuronal marker Tau was not detected in its phosphorylated form. Without specific phenotype inducers, EGF-bFGF pre-treatment did not direct the neuronal differentiation toward a default neuronal pathway as mature neurotransmitter markers were only slightly detected. However, we previously demonstrated that MIAMI cells were able to differentiate toward the dopaminergic phenotype in response to the correct instructive cues sonic hedgehog, fibroblast growth factor 8 and retinoic acid (Tatard, D'Ippolito et al. 2007).

For central nervous system cell therapy purposes, it is important to ascertain that MIAMI cells not only acquire a neuronal phenotype but also loose their mesodermal differentiation potential. Importantly, in this study, differentiated MIAMI cells did not express osteogenic and adipogenic specific genes and furthermore the EGF-bFGF pre-treatment induced a decreased expression of Sox9, present in chondrogenic progenitors (Marshall and Harley 2000). Only PPAR gamma, commonly related to the adipogenic and neurogenic lineages (Wada, Nakajima et al. 2006; Casteilla, Cousin et al. 2007; Shiota, Heike et al. 2007; Cimini and Ceru 2008) was up-regulated during differentiation. Indeed, the expression of the three PPAR variants have also been described in neural stem cells and PPAR gamma is now known to play a role in the regulation of neural stem cells proliferation and differentiation (Wada, Nakajima et al. 2006; Cimini and Ceru 2008). Moreover, lipid droplets were never observed inside the cells, unless if differentiated in a lipid rich medium supplemented with insulin, transferrin and selenium, commonly used for neuronal differentiation purposes (Okabe, Forsberg-Nilsson et al. 1996; Sanchez-Ramos, Song et al. 2000; Levy, Merims et al. 2003; Tao, Rao et al. 2005; Song, Song et al. 2007). PPAR gamma expression and the ability to uptake lipids are also observed in oligodendrocyte cell lines (Roth, Leisewitz et al. 2003). In this regard, the increased ability of differentiating EGF-bFGF pre-treated cells to produce lipid vesicles may be related to the observed up-regulation of the oligodendrocyte specific gene GalC even if Olig 2, mainly involved in the development of oligodendrocyte lineage (Jakovcevski and Zecevic 2005), was never detected. In addition, Notch1 down-regulation renders possible the oligodendrocytic differentiation of NSCs (Wang, Sdrulla et al. 1998), so this may suggest MIAMI cells commit toward a neuronal and preoligodendrocytic lineage after EGF-bFGF pre-treatment. In this respect, it has been proposed that some neurons and oligodendrocytes may share common progenitors during central nervous system development (Rowitch, Lu et al. 2002).

In conclusion, this simple EGF-bFGF pre-treatment of MIAMI cells started to specify the cells toward the neural/neuronal lineage and improved their neuronal differentiation program *in vitro*. In this regard, other expansion methods, involving for example culture in spheroids in an EGF-bFGF containing medium have been described prior to neuronal differentiation, but many of the cells died in the process of sphere formation (Hermann, Gastl et al. 2004)**.** These studies show that human MSCs may constitute an interesting source of cells for cell therapy of the nervous system. Importantly, MIAMI cells isolated from the iliac crest of living donors exhibited similar responses to EGF-bFGF pre-treatment than MIAMI cells isolated from vertebral bodies, therefore allowing a simplified harvesting for autologous transplantation in clinical protocols.

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DISCLOSURE OF INTERESTS

There is no disclosure of interest in this publication.

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CONCLUSION

Ce simple traitement en EGF-bFGF utilisé pendant l'expansion des cellules MIAMI à permis d'orienter la spécification des cellules vers un phénotype neuronal. Ce phénomène s'est traduit par une baisse de la prolifération des cellules, une baisse d'expression d'Oct4a, un facteur de transcription considéré comme un marqueur de cellules souches pluripotentes (Liedtke, Stephan et al. 2008) ainsi que par des changements d'expression de gènes neuraux précoces, caractéristiques d'une spécification neuronale. Ainsi, nous avons observé une baisse de *Notch1*, dont l'activation permet de maintenir les CSN en prolifération dans un état non-différencié (Kageyama, Ohtsuka et al. 2005). L'activation de *Notch1* active normalement la transcription de *HES5* qui interdit la transcription de *Ngn2*, un marqueur neural précoce. En outre, dans cette étude, la baisse de *HES5* et l'augmentation de *Ngn2* observées étaient cohérentes avec la baisse de *Notch1*. Enfin, une augmentation de la Nestine ainsi que de *Pax6*, marqueurs importants dans le mécanisme de différenciation neuronale des CSN (Kallur, Gisler et al. 2008) ont été observées suite au pré-traitement en EGF-bFGF.

Ce pré-traitement a également induit une augmentation des récepteurs au « nerve growth factor » (NGF) et au NT3 (NTRK1 et NTRK3, respectivement). Une phosphorylation d'ERK plus longue, un élément de la voie de signalisation en aval de NTRK3, fut également observée en réponse à une stimulation par le NT3. Ces résultats suggèrent que le pré-traitement en EGF-bFGF peut favoriser la réponse ultérieure des cellules soumises au protocole de différenciation *in vitro*.

Ce potentiel de différenciation neuronale *in vitro* fut effectivement nettement amélioré suite à ce pré-traitement, avec une importante baisse de la prolifération en cours de différenciation, confirmée par la forte expression de *p21* et la faible expression de la *Cycline D1*, deux marqueurs du cycle cellulaire. L'expression de marqueurs neuraux et neuronaux tels que la β3-Tubuline et des neurofilaments était aussi cohérente avec un phénomène de différenciation neuronale. La morphologie des cellules différenciées suite au pré-traitement a été enfin nettement améliorée, avec des cellules de plus de 400 µm de long, possédant un corps cellulaire fin avec de très longs prolongements, semblables à la morphologie neuronale.

Cette étude démontre une fois de plus le potentiel des CSM humaines MIAMI pour la thérapie cellulaire/ingénierie tissulaire du SNC. Notons que des cellules MIAMI issues de la moelle osseuse de 2 origines différentes furent utilisées dans cette étude (i.e. de vertèbres et de la crête iliaque de donneurs vivants) et se comportèrent de façon très similaires. Dans un contexte clinique, les cellules MIAMI issues de la crête iliaque pourraient être utilisées préférentiellement lors de greffe autologues en raison de leur facilité d'accès.

CHAPITRE 4

Microcarriers pharmacologiquement actifs enrobés de laminine et transportant des cellules stromales mésenchymateuses humaines pour protéger les neurones dopaminergiques dans la maladie de Parkinson

INTRODUCTION

Les microcarriers pharmacologiquement actifs (MPA), adaptés pour une utilisation avec les cellules stromales mésenchymateuses humaines, peuvent-ils améliorer le comportement de rats hémi-parkinsoniens ?

Ce chapitre décrit l'utilisation de MPA, un outil d'ingénierie tissulaire ayant recours à l'approche biomimétique conjointement avec la libération prolongée d'un facteur thérapeutique, dans le but d'améliorer la survie des cellules MIAMI, leur capacité de réparation tissulaire après transplantation, mais également afin d'orienter davantage celles-ci vers un phénotype neuronal. Ainsi, des cellules MIAMI pré-traitées en EGF-bFGF et induites vers un phénotype dopaminergique seront mises en contact avec des MPA et transplantées afin d'évaluer la réparation neuronale dans un modèle animal de la maladie de Parkinson.

Les preuves de ce concept ont précédemment été apportées dans un modèle de rats hémi-parkinsoniens avec des MPA transportant des cellules PC12 et libérant du NGF (Tatard, Venier-Julienne et al. 2004), mais également avec des cellules du mésencéphale ventral attachées à des MPA libérant du GDNF (Tatard, Sindji et al. 2007). Dans les études décrites précédemment, les MPA étaient recouverts d'une surface biomimétique constituée de FN et de poly-D-lysine (PDL), cette dernière étant nécessaire afin de permettre l'adhésion des cellules via les groupements chargés positivement des résidus lysines.

Un substrat de FN était également utilisé pour différencier les cellules MIAMI lors du protocole de différenciation neuronale en 3 étapes décrit dans le chapitre précédent. *In vivo*, les quantités de FN et de LM dans le cerveau augmentent dans le contexte de traumatismes crâniens, suggérant un rôle de ces molécules sur la réparation du tissu cérébral (Tate, Tate et al. 2007). Cependant, de nombreuses études décrivent désormais les bénéfices potentiels plus spécifiquement de la LM dans un contexte neuronal. Ainsi, le rôle de la LM pour la régénération axonale du SNC a été démontré *in vivo* (Grimpe, Dong et al. 2002). Une meilleure réparation tissulaire a également été obtenue dans un modèle murin de lésion corticale après transplantation de « scaffolds » composés de gel d'acide hyaluronique contenant de la LM (Hou, Xu et al. 2005). Dans un modèle *ex-vivo* de coupes cérébrales, l'utilisation de la LM semble également favoriser l'intégration et la migration de greffes de cellules embryonnaires transplantées ainsi que la régénération tissulaire (Kearns, Scheffler et al. 2006). Quelques études menées avec les CSM indiquent que la LM peut également avoir des effets bénéfiques sur leur différenciation neuronale, en termes d'expression de marqueurs neuraux mais également de morphologie *in vitro*. Ainsi, une équipe a observé une expression

de la Nestine sur des CSM cultivées en présence de LM 111 (Ho, Yu et al. 2006), et d'autres ont constaté une ramification plus importante des prolongements cellulaires sur un substrat de LM *in vitro* (Qian and Saltzman 2004), suggérant ainsi une activité biologique de cette protéine de la MEC sur la différenciation neuronale de CSM.

Pour ces raisons, nous avons caractérisé la présence d'intégrines capables de se lier à la FN et à la LM à la surface des cellules MIAMI. En effet, les intégrines sont une classe importante de récepteurs membranaires, dont une fraction est capable de se lier à ces molécules de la MEC en induisant un signal intracellulaire. Ces signaux intracellulaires ont pour origine le site d'adhésion focal, défini comme étant la partie de la cellule en contact direct avec la MEC (Hynes 2002). La protéine Src est localisée au niveau de ce site d'adhésion focal et est en contact direct avec l'extrémité intracytoplasmique des intégrines. C'est par conséquent l'une des premières protéines à pouvoir être phosphorylée lors de l'interaction entre intégrines et molécules de la MEC (Tucker, Rahimtula et al. 2008). Nous avons donc étudié la phosphorylation de Src sur un substrat de LM par western blot afin de vérifier que la LM activait effectivement une voie de signalisation intracellulaire chez les cellules MIAMI. Nous avons ensuite étudié l'effet de la LM sur les cellules MIAMI au cours de la différenciation neuronale, en termes de vitesse de prolifération, de morphologie ainsi que d'expression de marqueurs neuraux et neuronaux.

Nous avons finalement adapté la surface biomimétique des MPA par une utilisation combinée de PDL et de LM (MPA-LM), tout en veillant à garder une charge de surface similaire à ce que l'on avait précédemment observé avec la surface de FN/PDL. Cette nouvelle surface biomimétique de LM fut caractérisée en termes de charge de surface ainsi que par immunofluorescence afin d'estimer qualitativement l'homogénéité du recouvrement de la surface. Les conditions optimales nécessaires à l'adhésion des cellules, notamment le ratio MPA/cellules furent également étudiées.

Le but principal de cette étude était de caractériser l'effet des greffes de complexes MPA-LM/cellules MIAMI dans un modèle de lésion partielle de rats hémi-parkinsoniens (Kirik, Rosenblad et al. 1998). Ce type de lésion striatale est plus proche des stades précoces de la maladie de Parkinson qu'une lésion totale effectuée au niveau de la voie nigro-striée ou de la substance noire, en raison de son caractère partiel et évolutif au cours du temps. De plus, un tel modèle autorise l'observation d'un effet des greffes sur la repousse des fibres dopaminergiques survivantes. Les cellules transplantées peuvent en effet contribuer à la neuroprotection et à la neuroréparation du striatum lésé grâce à leur potentiel de différenciation en cellules de phénotype neuronal dopaminergique mais également grâce à la

sécrétion de divers facteurs de croissance et de chimiokines. La combinaison avec les MPA pourrait permettre d'augmenter la survie des cellules MIAMI tout en guidant leur différenciation neuronale. Pour cela, les cellules MIAMI ont été prétraitées en EGF-bFGF suite aux résultats du chapitre 3, puis exposées à l'effet de molécules inductrices d'un phénotype dopaminergique au cours du protocole de différenciation décrit précédemment (Tatard, D'Ippolito et al. 2007), avant d'être mises en contact avec les MPA-LM. L'effet de ces greffes a été évalué par le test de rotation induite par les amphétamines pendant 8 semaines.

Dans ce modèle animal de la maladie de Parkinson (Kirik, Rosenblad et al. 1998), la lésion striatale d'un seul hémisphère engendre un comportement rotatoire ipsilatéral en réponse à une stimulation par les amphétamines, dont l'intensité varie en fonction de l'étendue de la lésion. Enfin, l'étude de la survie, de la différenciation des cellules et l'effet sur la protection/réparation du système nigro-strié 8 à 9 semaines après transplantation est actuellement en cours.

En raison de la dépendance des cellules MIAMI au NT3 pour se différencier en cellules de phénotype neuronal (Tatard, D'Ippolito et al. 2007), cette neurotrophine apparaît comme une candidate idéale pour une encapsulation au sein des MPA-LM transportant les cellules MIAMI pour la réparation du système nigro-strié lésé. Après implantation dans les rats hémi-parkinsoniens, le NT3 sera libéré *in vivo* et pourra agir en plus de la surface de LM afin d'améliorer la survie et la différenciation des cellules MIAMI transportées. Ce facteur neurotrophique pourra aussi agir directement sur la réparation du tissu lésé. L'étude des effets de greffes de MPA-LM transportant des cellules MIAMI et contenant du NT3 est actuellement en cours.

Laminin-coated pharmacologically active microcarriers conveying human multipotent mesenchymal stromal cells protect the lesioned nigro-striatal system leading to functional improvement in hemiparkinsonian rats

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Running title: PAMs and MIAMI cells for Parkinson's disease

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ABSTRACT

Multipotent marrow stromal cells (MSCs) raise great interest for brain cell therapy due to their ease of isolation from bone marrow, their immunomodulatory potential, and their ability to differentiate into neural-like cells. In this study, we assessed the benefits of a tissue engineering strategy combining a subpopulation of human MSCs, the marrow-isolated adult multilineage inducible (MIAMI) cells, with pharmacologically active microcarriers (PAMs) to further improve cell behaviour leading to functional recovery in a rat model of Parkinson's disease (PD). PAMs are biodegradable and biocompatible poly(lactic-co-glycolic acid) (PLGA) microspheres with a biomimetic surface conveying cells on their surface, therefore providing an adequate 3D microenvironment *in vivo*. Moreover, they release a growth factor in a prolonged manner. These combined properties may act together to stimulate the survival and/or differentiation of the grafted cells toward a specific phenotype.

In order to define the biomimetic surface of the PAMs, the expression of a chosen panel of integrins by the MIAMI cells was evaluated. The results obtained prompted us to compare the effects of laminin (LM) and fibronectin to further enhance the *in vitro* neuronal differentiation of MIAMI cells. We demonstrated that LM diminished the proliferation, enhanced neurite length and neural/neuronal marker expression of MIAMI-derived neuronal-like cells. The PAMs were thus formulated with a LM biomimetic surface. This surface was then characterized in terms of zeta potential and immunofluorescence to assess and optimize the homogeneity of LM adsorption around the microspheres and their cell adhesion capacity.

After adhesion of dopaminergic-induced (DI)-MIAMI cells to LM-coated PAMs *in vitro*, the complexes were grafted in the partially dopaminergic deafferented striatum of rats. Amphetamine-induced rotational behaviour was strongly decreased when PAMs were used in combination with DI-MIAMI cells, whereas cells alone did not demonstrated major benefits compared to sham-treated rats. This functional effect resulted from the protection/repair of the nigro-striatal pathway, most probably due to an improved cell survival, and/or neuronal differentiation, upon cell attachment onto LM-PAMs surface. To our knowledge, this is the first study combining adult stem cells and tissue engineering in rat models of PD. After further characterization of the underlying mechanisms, this strategy may ultimately set the ground for pre-clinical studies with non-human primates.

Keywords: pharmacologically active microcarriers, laminin, mesenchymal stromal cells, MIAMI cells, cell therapy, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD), characterized by the loss of striatal dopaminergic fibres, is a progressive neurodegenerative disorder that affects 2 % of the population over 65 years of age. The most current and efficient therapeutic treatment, L-DOPA, aims at replenishing the amount of dopamine missing in the striatum. However, this strategy slowly becomes less effective after long-term treatment and shows undesirable side effects (Papa, Engber et al. 1994; Fahn 1996). Cell therapy is an alternative strategy to treat PD and many clinical studies using foetal dopaminergic cells or dopamine producing cells, such as adrenal chromaffin cells or human retinal pigment epithelium (hRPE) have been performed (Delcroix, Schiller et al., under review)(Lindvall and Hagell 2002; Drucker-Colin and Verdugo-Diaz 2004). These studies gave encouraging results that have provided the proof of principle for cell therapy in PD. However, the outcome was also highly variable between patients and the foetal grafts raised ethical and practical concerns (Lindvall and Kokaia 2009). Indeed, foetal ventral mesencephalic (FVM) cells, commonly transplanted in the context of PD, results in a limited host reinnervation and up to 90 % of the transplanted dopaminergic neurons die after grafting (Brundin, Barbin et al. 1985; Kordower, Rosenstein et al. 1996; Kordower, Freeman et al. 1998; Schierle, Hansson et al. 1999). Therefore, around 4 foetuses are necessary to ensure a clinical benefit highlighting the availability problem. In this regard, the identification of alternative sources of cells for cell therapy in the central nervous system is of great importance.

Stem cells, that can self renew and further differentiate into dopaminergic precursors are currently the most studied candidates for cell therapy in PD. Due to the difficulties in obtaining neural stem cells from adults and the inherent ethical problems to the use of foetal NSC or of embryonic stem cells, multipotent mesenchymal stromal cells (MSCs), may represent an alternative cell source to repair the nervous system (Delcroix, Schiller et al., under review). Indeed, as they are isolated from the bone marrow, autologous grafts can be performed avoiding ethical and availability concerns. MSCs may differentiate into progeny of the three embryonic layers *in vitro*, including neural-like cells, under the influence of matrix molecules and growth factors (Delcroix, Curtis et al., under review)(Song and Sanchez-Ramos 2003; Hermann, Liebau et al. 2006; Ross and Verfaillie 2008). Using appropriate driving cues, these cells may also be directed toward a dopaminergic phenotype (Tatard, D'Ippolito et al. 2007; Trzaska, Kuzhikandathil et al. 2007; Barzilay, Kan et al. 2008), therefore being of interest for PD cell therapy. In addition to their neuronal differentiation

potential, they posses immunomodulatory properties (Le Blanc 2003; Nasef, Mathieu et al. 2007) and have the ability to migrate towards sources of lesions in the brain (Mahmood, Lu et al. 2002; Jendelova, Herynek et al. 2004; Hellmann, Panet et al. 2006; Sykova and Jendelova 2007a; Delcroix, Jacquart et al. 2009). Furthermore, recent studies showed a functional improvement in the rotational behaviour of hemi-parkinsonian rats upon transplantation of rat or human MSCs (Bouchez, Sensebe et al. 2008; Levy, Bahat-Stroomza et al. 2008; McCoy, Martinez et al. 2008; Sadan, Bahat-Stromza et al. 2009). As a very small number of neuronal-like cells were observed in the brain (Levy, Bahat-Stroomza et al. 2008), these effects were mostly attributed to their ability to secrete various growth factors. These reports encourage further studies with MSCs for cell therapy of PD, but also highlight the need to enhance MSC cell engrafment.

Tissue engineering, which combines cells with a supportive scaffold providing a 3D structure, may help to improve cell engrafment after transplantation (Orive, Anitua et al. 2009). In this way, microcarriers transporting FVM cells or adrenal chromaffin cells can improve their long term survival after intracerebral transplantation in hemiparkinsonian rats (Cherksey, Sapirstein et al. 1996; Saporta, Borlongan et al. 1997; Borlongan, Saporta et al. 1998). A clinical trial has also reported the safety and efficacy of gelatine microcarriers conveying human retinal pigment epithelial cells for the treatment of PD (Bakay, Raiser et al. 2004; Stover and Watts 2008). Scaffolds providing a biomimetic surface of different ECM molecules or their derived peptides, that stimulate cell survival and differentiation, may further enhance cell engrafment (Tatard, Menei et al. 2005)(Delcroix, Schiller et al., under review). In this regard, various studies report that LM enhances neurite outgrowth of neurons (Tucker, Rahimtula et al. 2005) as well as NSC proliferation (Hall, Lathia et al. 2008). In addition, LM may also improve the integration of transplanted cells and their tissue regeneration potential in an *ex vivo* model of Parkinson's disease (Kearns, Scheffler et al. 2006). Finally, LM is known to affect stem cell motility (Kearns, Laywell et al. 2003) and differentiation of MSCs towards a neuronal phenotype, in terms of morphology (Qian and Saltzman 2004) but also of marker expression (Ho, Yu et al. 2006).

By combining *in situ* controlled drug delivery and biomaterial-based scaffolds, we have developed an adaptable and efficient device for tissue engineering, the pharmacologically active microcarriers (PAMs). They are biodegradable and biocompatible polymeric microcarriers made of poly(lactic-co-glycolic acid) (PLGA), that with a functionalized surface can provide an adequate 3D support for cell culture and/or for their administration. Their microcarrier role, the biomimetic surface and the programmed delivery

of an appropriate therapeutic factor may act synergistically to induce and further maintain the survival and/or differentiation of the transplanted cells, therefore enhancing their engraftment after complete degradation of the vector. Moreover the delivered factor may also modify the microenvironment (Tatard, Venier-Julienne et al. 2005). Thus, depending on the choice of the encapsulated factor and of the molecules adsorbed on their surface, PAMs may be customized for various cell types and applications in cell therapy. The efficacy of this tool was demonstrated in a rat PD paradigm using PAMs conveying PC12 cells and releasing nerve growth factor (Tatard, Venier-Julienne et al. 2004), but also with FVM cells attached to PAMs releasing glial cell line-derived neurotrophic factor (Tatard, Sindji et al. 2007). In both cases, the PAMs stimulated cell survival and differentiation leading to an improved behaviour of the animals.

The goal of the present study is to implement PAMs with a LM biomimetic surface (LM-PAMs) for transplantation of human MSCs in a rat model of PD. We used a homogeneous and unique subpopulation of human MSCs that express pluripotent stem cells markers. These cells named “marrow-isolated adult multilineage inducible” (MIAMI) cells, may generate mature cells derived from all three embryonic germ layers (D'Ippolito, Diabira et al. 2004; D'Ippolito, Diabira et al. 2006). We have shown that MIAMI cells on a FN surface differentiate towards immature neurons with neuronal ionic channel activity in a neurotrophin-3 (NT3)-dependent manner (Tatard, D'Ippolito et al. 2007). Moreover, exposing the cells to an EGF and bFGF pre-treatment enhances the neural specification and response to neuronal commitment of MIAMI cells (Delcroix, Curtis et al., under review). In order to choose the appropriate biomimetic surface, we first screened the panel of integrins expressed by MIAMI cells by RT-qPCR and flow cytometry. We then studied the effect of LM, compared to FN, on the *in vitro* neuronal differentiation of MIAMI cells in terms of cell proliferation, cell length and expression of neural and neuronal markers. PAMs with a biomimetic surface made of LM and PDL were formulated and their total charge as well as the homogeneity of the LM biomimetic surface were characterized by zetametry and immunofluorescence imaging. We then defined optimal conditions for MIAMI cell adhesion on PAMs prior to transplantation into the rat brain. Indeed, the main goal of this study was to further improve dopaminergic-induced (DI)-MIAMI cells survival, differentiation and tissue repair function after implantation in the striatum of hemiparkinsonian rats, using PAMs tailored for this application. Therefore, LM-PAMs/DI-MIAMI cells complexes were obtained and their effects on animal behaviour, tissue repair/protection, as well as the cells’ behaviour after implantation were evaluated in a rat partial and progressive model of PD.

MATERIALS AND METHODS

Bone marrow harvesting, selection & expansion of MIAMI cells. Whole bone marrow was obtained from vertebral bodies (T1–L5) of a 3 year old male cadaveric donor following guidelines for informed consent set by the University of Miami School of Medicine Committee on the Use of Human Subjects in Research. As previously described (D'Ippolito, Diabira et al. 2004), isolated whole bone marrow cells (referred to as MIAMI cells #519) were plated at a constant density of 10^5 cells/cm^2 in DMEM-low glucose, containing 5 % fetal bovine serum (FBS) (Hyclone, South Logan, Utah) and antibiotics (AB) (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, Sigma, St-Quentin Fallavier, France) on a FN (Sigma) substrate under hypoxic conditions (3 % O_2, 5 % CO_2, 92 % N_2). Fourteen days later, the non-adherent cells were removed and pooled colonies of adherent cells were selected and plated at low density for expansion (100 cells/cm²) on 1.25 ng/cm² FN substrate in DMEM-low glucose (Gibco, Cergy Pontoise, France) containing 3 % FBS and AB (40 mL/175 cm² flask) under hypoxic conditions. Cells were fed every 2-3 days by changing half the medium and split every 5 days, keeping 1/4 of old medium.

MIAMI cells neuronal differentiation. To assess the effects of the extracellular matrix molecules on the 3-step *in vitro* neuronal differentiation of MIAMI cells (previously described in Delcroix, Curtis et al. under review), MIAMI cells passage 4-5 were first expanded for 10 days in DMEM-low glucose with 20 ng/mL of both EGF and bFGF, 5 µg/mL heparin and a mixture of lipids (working concentration of 510 nM lipoic, 70 nM linolenic and 150 nM linoleic acid, all from Sigma) to enhance their specification and further commitment toward the neuronal lineage (pre-treatment step). Cells were then plated at 3000-4000 cells/cm^2 on either FN (#F1141, Sigma), LM (from human placenta, #L6274, Sigma) coated dishes (all at 2 µg/cm², unless otherwise stated) in DMEM-F12 (GIBCO) medium supplemented with 20 % FBS, 20 ng/mL of both EGF-bFGF, 5 µg/mL heparin, antibiotics and cultured for 24 h (Neural specification, step 1). Coating molecules were diluted in Dulbecco's phosphate buffered saline (DPBS) (w/o Ca and Mg) and coating was made in presence of 1 mM $CaCl_2$ for LM as it was previously reported to enhance its stabilization (Freire, Gomes et al. 2002). When plated on glass slides, LM coating was sometimes made at 0.5 µg/cm² as it allowed a sufficient attachment of cells during differentiation. At the end of step 1, cells were washed and neuronal commitment (step 2) was induced by exposing the cells to 1 mM β-mercaptoethanol (Sigma), 30 ng/ml NT3 (R&D Systems) for 2 days. Neuronal differentiation (step 3) was induced by washing and then exposing the cells to 100

µM butylated hydroxyanisole, 25 mM KCl, 2 mM valproic acid, 4 µM forskolin, 1 µM hydrocortisone, 5 µM insulin, 5 mM Hepes, 4 µM forskolin, 10 µM rolipram (all from Sigma), 30 ng/mL NT3, 10 ng/mL NGF (R&D Systems), and 30 ng/mL BDNF (R&D Systems) for 3 days.

For transplantation, EGF-bFGF pre-treated cells were further induced toward a dopaminergic phenotype (DI-MIAMI cells) prior to their attachment to PAM's biomimetic surface. Pre-treated cells were plated (3000-4000 cells/cm²) on a 2 µg/cm² LM substrate in DMEM-F12 containing 20 % FBS, 20 ng/mL of both EGF-bFGF and 5 µg/mL heparin for 24 hours. Medium was then replaced by DMEM-F12 containing 200 ng/mL SHH and 100 ng/mL FGF8b for another 24 hours (both from R&D Systems). Finally, cells were exposed to 0.5 µM retinoic acid (Sigma) during the last 24 hours before attachment to PAMs. Dopaminergic phenotype induction was performed in a normoxic atmosphere.

Immunocytofluorescence (ICF). Cells were plated on coated glass slides for NFM and β3-Tubulin ICF staining at the end of the neuronal differentiation protocol. After washing twice with DPBS, cells were fixed with 4 % paraformaldehyde (PFA) (Sigma) at 4°C for 15 min and then permeabilized for 5 min with DPBS containing 0.2 % Triton X-100 (DPBS-T) (Sigma). Slides were blocked with DPBS, 10 % normal goat serum (NGS) (Sigma), 4 % bovine serum albumin (BSA) (Sigma) at room temperature (RT) for 45 min. After 3 washes in DPBS, slides were then incubated overnight at 4°C with 100 µg IgG1/mL of anti-NFM (clone NN18, Sigma) or 3.8 µg IgG2b/mL of anti-β3-Tubulin (clone SDL.3D10, Sigma) antibodies in DPBS-T-4 % BSA. Controls were made without primary antibody and with isotypic IgG1k (clone MOPC-31C, BD Biosciences) or IgG2bk (clone 27-35, BD Biosciences). After 3 further washes in DPBS, incubation with 2.5 µg/mL secondary biotinylated anti-mouse antibody (#BA2001, Vector, Burlingame, CA, USA) in DPBS-T-4 % BSA was made for 1 hour at RT. Finally, after washing 3 times in DPBS, incubation with 20 µg/mL streptavidin FITC (#F0422, Dako) in DPBS for 40 min at RT was made before mounting (Dako) and observation with a fluorescence microscope (Axioscop, Carl Zeiss, Le Pecq, France), a CoolSnap ES camera (Photometrics, Tucson, Arizona) and Metamorph™ software (Roper Scientific, Evry, France).

Real-time quantitative PCR. Design of primers specific for human genes and PCR were performed as described elsewhere (Delcroix, Curtis et al, under review) (table 7). Cells were lysed in a 1 % β-mercaptoethanol containing buffer and RNA extracted following treatment by DNAse to remove any traces of genomic DNA (Total RNA isolation Nucleospin® RNA II, Macherey Nagel, Hoerdt, France). First strand cDNA synthesis was performed with a Ready-

To-Go You-Prime First-Strand Beads® kit in combination with random hexamers (Amersham Biosciences, Orsay, France) using 1 µg RNA according to the manufacturer's guidelines. Following first strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France), eluted in 50 µL RNAse free water (Gibco). Five microliters of cDNA (1:20) were mixed with iQ SYBR Green Supermix (Biorad) and primer mix (0.2 µM) in a final volume of 15 µL. Amplification was carried on a Chromo4 thermocycler (Biorad) with a first denaturation step at 95°C for 3 min and 40 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 15 s.

Gene	Full name	NM accession number	Sequences	Amplicon
ITGB1	*Integrin B1*	002211-033666-033667-033669-033668-133376	F=5'-CGGACAGTGTGTTTGTAGG-3' R=5'-CAGTGTAGTTGGGGTTGC-3'	161
ITGB3	*Integrin B3*	000212	F=5'-TACAAACACGTGCTGACG-3' R=5'-GAGTCTTGGCATCAGTGG-3'	196
ITGB4	*Integrin B4*	000213-001005619-001005731	F=5'-CTGCACCTACAGCTACACC-3' R=5'-CACAGTACTTCCAGCATAGC-3'	173
ITGA1	*Integrin A1*	181501	F=5'-GGCCGTAGTTAAAGTGACC-3' R=5'-GTGAATCTAGGGTGACACG-3'	185
ITGA2	*Integrin A2*	002203	F=5'-TCCAGACAGTACAGCTAACG-3' R=5'-GCAACCAGAGCTAACAGC-3'	187
ITGA3	*Integrin A3*	005501-002204	F=5'-TGAGAACTTCTACCCAGACC-3' R=5'-GGCACTCTGGTTGTAAGC-3'	187
ITGA4	*Integrin A4*	000885	F=5'-CGCTTCAGTGATCAATCC-3' R=5'-CTGTCTGGAAAGTGTGACC-3'	166
ITGA5	*Integrin A5*	002205	F=5'-GAGCAGAACCATGTGTACC-3' R=5'-CAAAGTAGTCACAGCTCAGG-3'	181
ITGA6	*Integrin A6*	000210-001079818	F=5'-CCCAGATATTGCAGTTGG-3' R=5'-CTGAATCTGAGAGGGAACC-3'	200
ITGA7	*Integrin A7*	002206	F=5'-GACTCACTGCACTACTCAGG-3' R=5'-CAGCTCTACCTCCAGTTCC-3'	192
ITGA8	*Integrin A8*	003638	F=5'-GAGATTCGGTAGTGCTATGG-3' R=5'-ACTCCTTGCAGAACTTGG-3'	168
ITGA9	*Integrin A9*	002207	F=5'-ACTGGAGAGGAGGAGAGG-3' R=5'-CCCCAAAGCTAGATACAGG-3'	195
ITGA11	*Integrin A11*	001004439-012211	F=5'-CATCCTGAAGACACCTAAGC-3' R=5'-CTGGCATTGATCTGAACC-3'	183
ITGAV	*Integrin AV*	002210	F=5'-CAGTCCCATCTCAAATCC-3' R=5'-CTGGCCCTGTATAAGATAGC-3'	160
*Nurr1 (NR4A2)**	*nuclear receptor subfamily 4, group A, member 2*	006186	F=5'-TTGCCAGATGCGCTTCGACG-3' R=5'-CCAACAGCCAGGCACTTCTG-3'	414
*DAT**	*Dopamine transporter*	001044	F=5'-GAAGGTGGTATGGATCACAG-3' R=5'-GTAGAAGTCAACGCTCAGGT-3'	121
*TH**	*Tyrosine hydroxylase*	199292-000360-199293	F=5'-CACCATCTAGAGACCCGGCC-3' R=5'-GCAATCAGCTTCCTGCGCTG-3'	290

Table 7. Human specific primer sequences

*Primers ordered from Origen, USA

Integrin screening by flow cytometry. Cells were washed with DMEM-low glucose and detached with 10 mL Versene (Lonza) for 20 min at 37°C. After pelleting at 295 g for 10 min, cells were washed twice before distribution in 296 well plates (10^5 cells/50 µL). 50 µL of 10 µg/mL mouse monoclonal anti-integrin antibodies (#555442, 555615, 556024, 555497 for CD29, CD49e, CD49c and CD49b respectively, all from BD Biosciences, Le Pont De Claix, France) or IgG1k isotypic antibody (#557273, BD Biosciences) solutions diluted in DPBS,

5 % FBS, 0.02 % azide were added and incubated for 1 h at 4°C. After washing, 20 µg/mL FITC-conjugated anti-mouse antibody (#F0479, Dako, Trappes, France) was added for 30 min at 4°C. Cells were rinsed 3 times before a final addition of DPBS, 0.02 % azide and transfer to tubes containing DPBS, 0.02 % azide, 0.7 % formaldehyde. Every washing step was performed with DPBS, 5 % FBS, 0.02 % azide. Fluorescent signal was acquired using a FACScalibur flow cytometer (BD Biosciences) and data analysis was performed using the Cellquest™ software (BD Biosciences). Isotypic control fluorescent signal geometric mean was always subtracted to the marker fluorescent signal geometric mean.

Microsphere formulation. A PLGA copolymer (Phusis, Saint Ismier, France) was used to prepare microspheres of 60 µm in diameter. The composition of the chains was 37.5 % D-lactic units, 37.5 % L-lactic units, and 25 % glycolic units (PLGA 37.5/25) PLGA molecular weight was 25,000 Da (I=1.8) as determined by size exclusion chromatography. Microspheres were prepared using the s/o/w emulsion solvent extraction-evaporation process described in (Giteau, Venier-Julienne et al. 2008b) with modifications. Briefly, an organic solution (2 ml; 3:1 methylene chloride:acetone) containing 150 mg of PLGA was emulsified in a poly(vinylalcohol) (Mowiol® 4-88, Kuraray Specialities Europe, Frankfurt, Germany) aqueous solution (90 ml, 4 % w/v) maintained at 1°C and mechanically stirred at 550 rpm for 1 min (Heidolph, RZR 2041, Merck Eurolab, Paris, France). After addition of 100 ml of deionized water and stirring for 10 min, the resulting o/w emulsion was added to 500 mL deionized water and stirred for a further 20 min to extract the organic solvent. Finally, the microparticles were filtered on a 0.45 µm filter (HVLP type, Millipore SA, Guyancourt, France), washed five times with 100 ml of deionized water and freeze-dried. The average volume diameter and the size distribution of the resulting microspheres were evaluated using a Multisizer™ Coulter Counter (Beckman Coulter, Roissy, France). For zeta potential measurements, microspheres with a reduced diameter (mean of 25 µm) had to be formulated. We produced these microspheres using the previous protocol with increased poly(vinylalcohol) concentration (6 % w/v) and agitation speed (1000 rpm).

Formulation of PAMs. To obtain PAMs, microspheres were coated with either FN or LM. A combination of LM or FN with the highly charged PDL (#P6407, Sigma) molecules was used to favour cell attachment on PAM's surface. Microspheres were resuspended in DPBS and sonicated upon full dispersion of the microspheres. Coating solutions were prepared in DPBS, mixed to the microsphere suspension and placed under rotation at 15 rpm at 37 °C during 4 hours. After coating, PAMs were washed 3 times in distilled sterile water, lyophilized and finally kept at -20°C for long term storage. Every tube was covered with sigmacote ® (Sigma)

to prevent product loss on the tube walls. The final concentration of the coating molecules was 40 µg/mL-60 µg/mL of FN, LM or a mixture of PDL/FN and PDL/LM. For optimization of the PDL/LM coating, the ratio PDL-LM of 60/40 was changed to 40/60 after zeta potential evaluation, in order to maximize the quantity of LM adsorbed to the surface. The incubation time was finally decreased to 1.5 hours as well as the total coating molecules concentration (15 µg/mL).

Zeta potential. A Zetasizer 2000 (Malvern Instruments, Orsay, France) operating at 150 V at RT was used to assess PAM's electrical surface charge variations depending on the coating used. Briefly, PAMs were redispersed in 10 mL of 1 mM NaCl and sonicated prior to every measurement. The chamber was washed with ultrapure water (Millipore) between every sample. Results are presented as the average of 10 measurements and experiments were performed in triplicate.

PAMs immunofluorescence. Tubes containing 1 mg coated or uncoated lyophilized PAMs were resuspended in DPBS, 4 % BSA, 0.2 % Tween 20 and placed for 30 min at RT under 15 rpm rotation. Samples were then washed three times with DPBS followed by a centrifugation step at 9000 g for 5 min and supernatant removal. Incubation with mouse monoclonal anti-laminin antibody (Ref L8271, Sigma) diluted at 100 µg IgG/mL in DPBS, 4 % BSA, 0.2 % Tween 20 was performed at 37°C for 1.5 hours under rotation. For negative controls, incubation was performed in the same solution without anti-laminin antibody. After incubation, samples were washed again 4 times before incubation with anti-mouse biotinylated antibody (#BA2001, Vector) diluted at 2.5 µg/mL in DPBS, 4 % BSA, 0.2% Tween 20 for 1 hour at RT under rotation. After three washes, samples were incubated with streptavidine-fluoroprobe 547 (Interchim, Montluçon, France) diluted 1:500 in DPBS for 40 min at RT under rotation. Samples were finally washed three times with DPBS and observed under confocal microscopy (Olympus Fluoview^{TM} TU 300, Rungis, France). Every condition was observed in triplicate and 3 independent experiments were performed.

Formation of PAM/DI-MIAMI cells complexes. DI-MIAMI cells were washed with DMEM-F12, detached with 0.16 % trypsin (Sigma), 0.02 % EDTA (Lonza) solution, and pelleted at 295 g for 10 min. Cell pellets were resuspended in culture medium supplemented with 3 % FBS. 0.75 mg lyophilized microspheres were resuspended in coated Eppendorf tubes (Sigmacote, Sigma) containing DMEM-F12, 3 % FBS for 15 min. PAMs suspension was sonicated and briefly vortexed prior to addition of 0.5 mL cell suspension ($2x10^5$ cells/0.75 mg PAMs). The mixture was then gently flushed and plated in 1.9 cm² Costar ultra low cluster plate (#3473, Corning, Avon, France). Plates were incubated at 37°C during 4

hours to allow cell attachment on PAM surface. PAMs/cell aggregates were recovered, washed with DMEM-F12 and pelleted by centrifugation at 200 g for 2 min. Cell adhesion to PAM's surface was assessed by microscopic observation and cells adhered to PAMs were quantified using Cyquant cell proliferation assay (Invitrogen, Cergy Pontoise, France), following the manufacturer's guidelines. A vehicle solution of CMC-Na-Tween 80-Mannitol (final concentration 0.125 %, 0.125 % and 0.5 %, respectively) was added to the aggregates in a final volume of 10 µL just prior to transplantation. To allow cell tracking after transplantation, DI-MIAMI cells were labeled with the membrane dye PKH26 (Sigma) before attachment to PAMs. Briefly, cells were washed with medium after harvesting and resuspended in 0.5 mL diluent C. PKH26 dye diluted in 500 µL diluent C (1 µL PKH26/ 1 million cells) was then added to the cell suspension and homogenized before incubation at 37°C for 5 min. Labeling reaction was stopped by washing three time with serum containing medium before proceeding to attachment onto PAMs.

Animals and surgical procedures. All animal experiments were conducted in accordance with the "Direction des Services Vétérinaires", the "Ministère de l'Agriculture" of France and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). A total of 50 female Sprague-Dawley rats, 12 week old and about 250 g in weight, were used in this study. Rats were anaesthetized with xylazine (7.7 mg/kg) and ketamine (41.5 mg/kg) and positioned in a Kopf stereotaxic instrument. Two injections of 10 µg of 6-hydroxydopamine (Sigma, France, in 5 µl saline supplemented with 0.1% ascorbic acid) were performed to induce a unilateral partial progressive and retrograde lesion of the nigro-striatal system, a lesion model previously described (Kirik, Rosenblad et al. 1998). The lesion coordinates were: (1) AP: 0.5, L: -2.5, and V: -5 mm, (2) AP: -0.5, L: -4.2, and V: -5 mm relative to bregma and ventral from dura, with the tooth-bar set at 0 mm. Only rats that showed more than 7, but less than 18 net ipsiversive turns per minute eleven days after the lesion were used. They were assigned to one of 3 treatment groups (5 animals per group): animals receiving intrastriatal injection of vehicle solution only (CMC-Na-Tween 80-Mannitol), DI-MIAMI cells alone and LM-PAMs conveying DI-MIAMI cells. On the day of transplantation, 2 weeks post-lesion, $1.5x10^5$ cells alone or attached to 0.75 mg of PAMs were stereotactically implanted with a 20 gauges Hamilton microsyringe (with a 25° bevel, to allow a better sample aspiration) in the lesioned striatum at the following coordinates: AP+0.5; ML–2.8; DV–5; tooth bar–3.3.

Quantification of human genomic DNA by real-time quantitative PCR analysis. Rats were sacrificed by CO_2 inhalation for quantification of human genomic DNA (gDNA). Striatum was extracted to obtain a sample of 100 mg and flash frozen in liquid nitrogen prior freezing at -80°C. Tissues were grinded on a mortar in presence of liquid nitrogen followed by gDNA extraction using Genomic Tip-100 columns and proteinase K, as described by the manufacturer (Qiagen, Courtaboeuf, France). DNA concentration and purity were determined by optical density using a Nanodrop ND 1000 spectrophotometer (Thermo scientific, Courtaboeuf, France). A human specific primer for β-globin was designed and validated for real-time quantitative PCR quantization of human gDNA. No amplification was observed with gDNA isolated from non-grafted rat striatum and positive sample fusion curve exhibited a thin and unique melting peak (Tm= 82°C). Forward primer was 5'-tgggcaggttggtatcaagg-3' and reverse primer 5'-gcctaagggtgggaaaatagacc-3'. PCR protocol was similar than for RNA amplification previously described, but with a first denaturing step of 10 min at 95°C and an annealing temperature of 60°C. Runs were performed with 100 ng of gDNA.

Behavioural study. Amphetamine-induced rotational behaviour was measured in an automated rotometer 11 days after the lesion and 2, 4, 6 & 8 weeks after grafting. The animals were weighed prior to each rotation test. All tests were conducted in a blind manner. 5 mg/kg of D-amphetamine (dissolved in NaCl 0.9%) (Sigma, France) was administered intraperitoneally. Animals were individually placed in circular plastic boxes and attached to the rotational leash 5 min before injection for accustoming. Immediately after amphetamine injection, the test began and the data were recorded for 90 min using a computerized system. A net rotational asymmetry score was expressed as full-body turns per minute in the direction ipsilateral to the lesion.

Histological study. Eight weeks after cell transplantation, the animals were anaesthetized and perfused through the heart with 150 ml of ice-cold 0.9 % saline, followed by 300 ml of ice-cold 4 % PFA, 2.5 % sucrose (Sigma) in DPBS pH 7.4. Brains were left 1.5 hours in the PFA solution and then transferred to 10 % sucrose solution. During the next 48 hours, the sucrose concentration was increased up to 30 %. Brains were finally frozen in cold isopentan (-40°C) before storage at -80°C. Striatal and substantia nigra sections, of 14 µm and 30 µm respectively, were made on a CM3050S cryotome (Leica Microsystems). Immunohistochemistry was used to assess the extent of the striatal lesion using a mouse anti-TH antibody (1:1000, clone 6D7, Covance, Emeryville, CA, USA). For the detection of DI-MIAMI cells, we used a mouse, human specific, anti-mitochondria antibody (1:100, clone MTCO2, Abcam, Paris, France) as well as a mouse anti-CD11b antibody (1:100, clone

MRCOX42, Abd Serotec, Cergy Saint-Christophe, France) (specific for macrophage-microglia) to assess the inflammatory reaction. Sections were firstly washed with DPBS-0.2 % Triton X-100. For TH staining, quenching of peroxidases was made with 0.3 % H_2O_2 (Sigma) in DPBS at RT for 15 min. After washing, blocking was performed with DPBS, 4 % BSA, 10 % NGS for 30 min at RT. Sections were incubated overnight at 4 °C with the primary antibodies diluted in DPBS-4 % BSA. After washing, sections were incubated at RT for 1 h with the secondary biotinylated anti-mouse antibodies (1:200, #BA2001, Vector) diluted in DPBS-4 % BSA. For TH staining, incubation with Vectastain® ABC reagent (Vector) in DPBS was made at RT for 1 h. Sections were washed and revealed with 0.03 % H_2O_2, 2 mg/mL diaminobenzidine (DAB) (Sigma) in DPBS and dehydrated before mounting. For human mitochondria and anti CD11b staining, sections were incubated with Streptavidine-Fluoroprobes 488 ® (1:200, Interchim) diluted in DPBS for 40 min at RT before mounting with fluorescent mounting medium. Free floating TH staining of substantia nigra was performed with a similar treatment than for striatal TH staining, appart from the use of a polyclonal rabbit anti-TH (1:20000, Jacques Boy, Reims, France) and of a biotinylated anti-rabbit secondary antibody (1:500, #BA1000, Vector).

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 a Student's t-test modified for small samples. $t'_0=(m_1-m_2)/\sqrt{(s_1^2/n_1+s_2^2/n_2)}$, differences were considered significant if $|t'_0|> t_{k':0.05}$, with k' being the closer integer of the calculated $k=(s_1^2/n_1+s_2^2/n_2)^2/[(1/(n_1-1)(s_1^2/n_1)^2)+(1/(n_2-1)(s_2^2/n_2)^2)]$. Threshold P-value was set to 0.05.

RESULTS

MIAMI cells integrin subunit screening

In preliminary experiments we observed a higher number of MIAMI cells expanded on FN compared to other substrates such as collagen (data not shown). This result together with the reported neuronal inducing effects of LM prompted us to screen the expression of integrins for these ECM molecules by MIAMI cells. During expansion of MIAMI cells, integrin subunits beta 1, alpha 2, 3, 5, 11 and V were highly expressed when evaluated by RT-qPCR. Subunits beta 3, 4 and alpha 1, 4, 6-9 were also detected but at much lower level (data not shown). The high expression of subunits beta 1, alpha 2, 3 and 5 was confirmed by flow cytometry (figure 14), thus making FN and LM adequate for PAM's biomimetic surface.

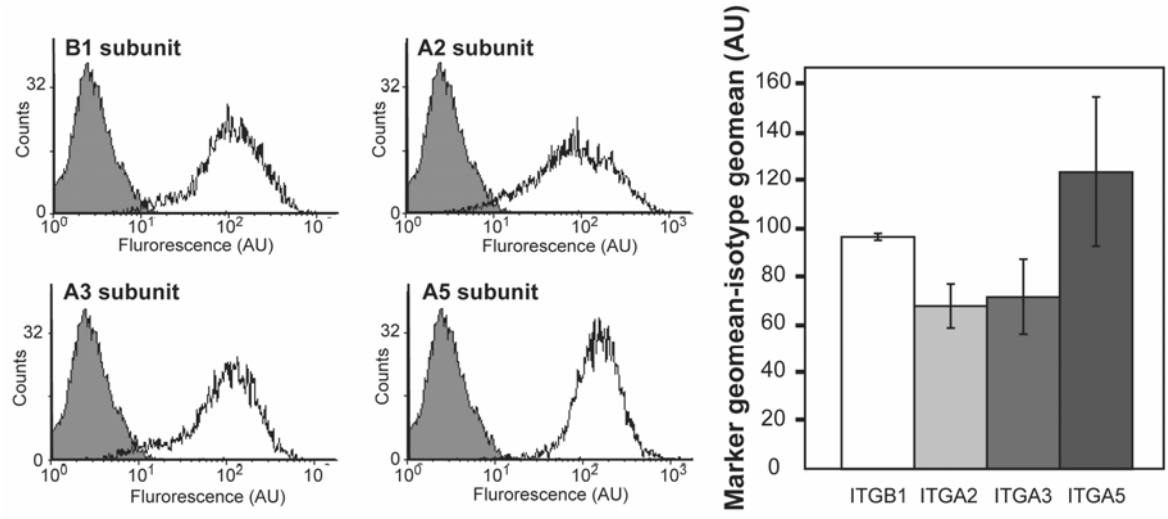

Figure 14. Flow cytometrical assessment of integrin (ITG) subunit expression

Expanding MIAMI cells strongly expressed the integrin subunits beta 1, alpha 2, 3 and 5, making both fibronectin and laminin suitable molecules for PAM's biomimetic surface. Top right histogram depicts results expressed as the geomean of the signal obtained with the anti-integrin antibodies minus the signal obtained with the corresponding isotypic antibodies.

In vitro neuronal differentiation on laminin vs fibronectin

We previously demonstrated (Delcroix, Curtis et al., under review) that during a neuronal induction protocol performed on a FN substrate, EGF-bFGF pre-treated MIAMI-derived neuronal-like cells showed a diminished proliferation rate, presented long neurites and acquired an expression pattern consistent with a neuronal differentiation program. Using Trypan blue counting, we observed that differentiating the EGF-bFGF pre-treated cells on a substrate of LM instead of FN led to a further decrease of cell proliferation that was abolished

most of the time (0.61 ± 0.06 doublings on FN *vs* 0.26 ± 0.05 doublings on LM) during *in vitro* neuronal differentiation. Importantly, dead cells were never observed during differentiation on either substrate. In addition to a decreased proliferation rate, total cell length was increased in most experiments at the end of differentiation, with longer neurites (figure 15A) when plated on 0.5 µg/cm² LM compared to glass or FN (figure 15B).

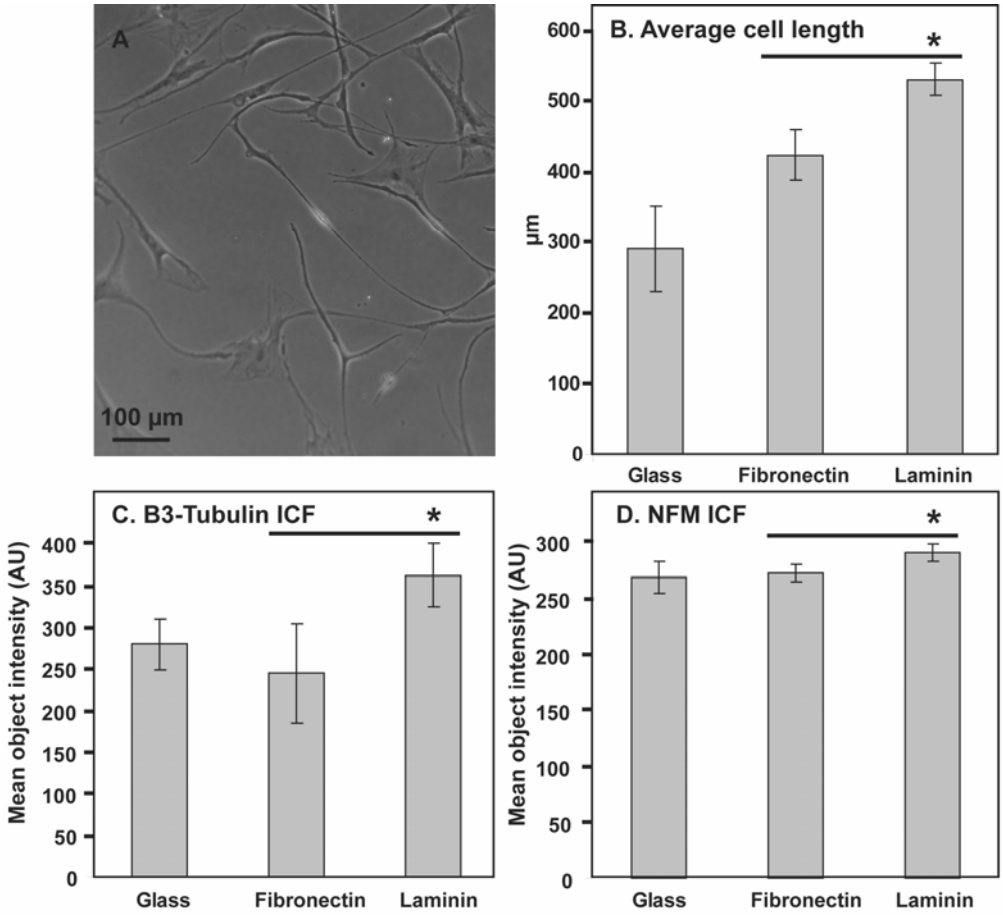

Figure 15. Benefits of laminin (LM) vs fibronectin (FN) during neuronal differentiation

LM enhanced neurite formation and resulted in an increased cell length at the end of step 3 (A, B). The expression of neural and neuronal markers, β3-Tubulin and NFM (C, D, respectively) were also enhanced at the end of step 3 using LM substrate, compared to FN or glass as observed by ICF. Cell length results are presented as the average length of the 3 longer cells of 5 different images ± average deviation and ICF results as the mean ± average deviation of averaged object intensities calculated from 6 different images. Depicted results were obtained from a representative experiment and differences considered significant at P=0.05 *.

Finally, we also observed by ICF that at the end of the induction protocol MIAMI-derived neuronal-like cells expressed higher levels of β3-Tubulin as well as, to a certain extent, NFM, when plated on 0.5 µg/cm² LM compared to glass or FN (figure 15C, D). We

can note that all these trends were also obtained with MIAMI cells not pre-treated with 20 ng/mL of EGF-bFGF, but to a much lesser extent (data not shown).

Characterization of PAMs' biomimetic surface

Uncoated PLGA microspheres, FN or LM-coated PAMs (60 µg/mL each) resulted in a strongly negative zeta potential (figure 16A), which was not satisfactory for cell adhesion. Conversely, PDL-coated PAMs exhibited a strongly positive Zeta potential (49.7 ± 2.1 mV). We therefore used a blend of PDL with FN or with LM to combine the benefits of a positively charged surface promoting cell adhesion and presenting ECM molecules. The association of FN or LM with PDL slightly decreased the zeta potential value compared to PDL alone. We next tested conditions allowing to optimize the ratio of PDL/LM, the concentration of coating molecules and the incubation time necessary for adsorption of the molecules on the surface. In this way, the ratio of LM was increased to 60 % instead of 40 %, the adsorption time decreased from 4 to 1.5 hours and the total quantity of coating molecules decreased from 40 µg/mL to 15 µg/mL. We next confirmed these optimized parameters did not impair the positive surface charge, which was 34.5 ± 2.6 mV (figure 16A, top right bar), and the homogeneity of the LM surface. Immunofluorescence imaging demonstrated that controls and uncoated microspheres, were negative (figure 16B, C) whereas the signal was intense and homogeneous all around the PAMs coated with a mixture of PDL/LM at a ratio of 40/60 (figure 16D, E). These optimized conditions were further used throughout this study.

In vitro characterization of LM-PAMs/DI-MIAMI cell complexes

For transplantation purposes a similar number of cells were grafted with or w/o PAMs. The number of cells adhering to PAMs was assessed using a Cyquant cell proliferation assay ®, and it was possible to graft $1.5x10^5$ cells adhered onto 0.75 mg of PAMs. Importantly, almost all the cells attached to the PAM's biomimetic surface after 4 hours at 37°C, and no free floating cells were observed (figure 16F). Moreover, gene expression of *Nurr1, DAT* and *TH* was not modified upon attachment to PAMs as observed by RT-qPCR. However, only basal levels of these markers were always detected in MIAMI cells, without significant differences upon early dopaminergic induction.

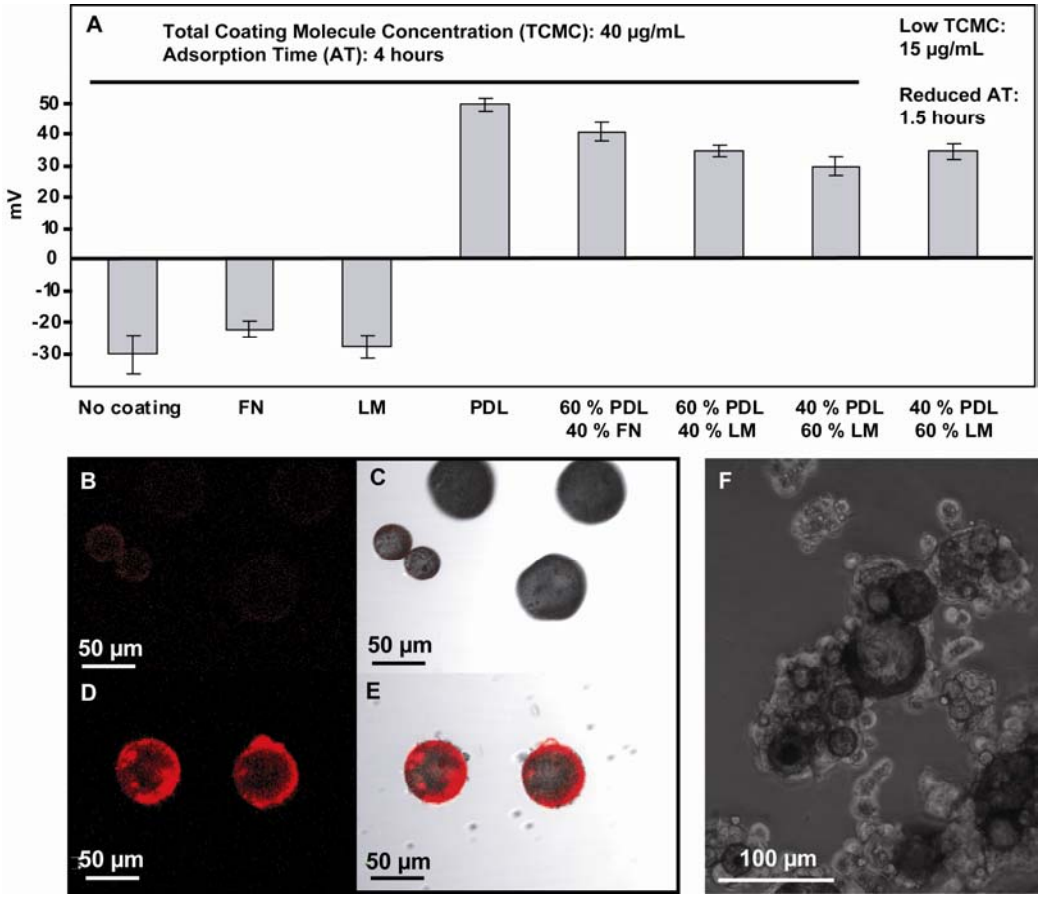

Figure 16. Characterization of PAM's biomimetic surface

Fibronectin (FN) and laminin (LM) induced a negative zeta potential whereas PDL shifted this potential toward positive values, favourable for cell attachment to the surface. Mixture of PDL/FN or PDL/LM resulted in a slightly reduced zeta potential compared to PDL alone. PDL/LM-coated PAMs formulation was optimized by using a ratio PDL/LM of 40/60 instead of 60/40 to increase the quantity of adsorbed LM (A). The adsorption time was decreased from 4 to 1.5 hours, and the total molecules concentration used for coating was decreased from 40 µg/mL to 15 µg/mL. No changes in Zeta potential were observed using these parameters that decreased formulation cost and minimized the possible loss of encapsulated protein during coating (A, top right bar). Using anti-LM immunofluorescence, uncoated microspheres (B, C) show no staining whereas PDL/LM-coated microspheres (D, E) stained positive with the anti-LM antibody. Importantly, LM was homogeneously distributed over the PAM's surface. C, E: superposition of Nomarsky and fluorescent images. After 4 hours attachment, almost all the cells adhered onto PAM's biomimetic surface made of PDL and LM (F) and it was possible to transplant $1.5x10^5$ cells attached to 0.75 mg of PAMs.

Behavioural study

Amphetamine-induced rotational behaviour of sham-treated rats continuously increased from the implantation day until the end of the experiment, at 8 weeks. Implantation of DI-MIAMI cells alone slightly reduced the number of ipsilateral rotations at late time points, but the difference was not statistically significant compared to sham-treated rats. However, transplantation of DI-MIAMI cells adhered onto LM-coated PAMs affected the rotational behaviour, which was strongly decreased compared to sham-treated rats or rats implanted with cells alone (figure 17). These observations show a behavioural improvement in rats treated with LM-PAMs/DI-MIAMI cells complexes.

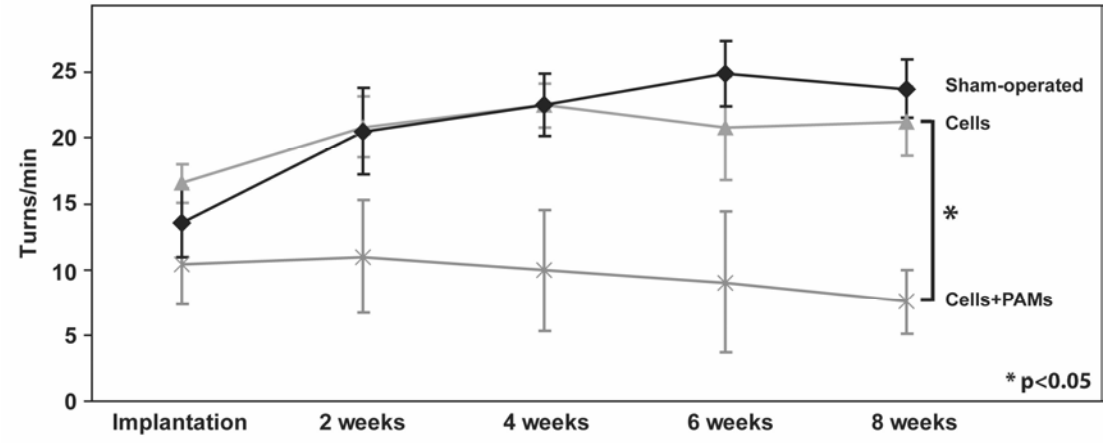

Figure 17. Amphetamine-induced rotational behaviour

Rats transplanted with medium alone (sham-operated) exhibited a constant increase of their rotational behaviour during 6 weeks before stabilization at 8 weeks. Rats transplanted with DI-MIAMI cells alone exhibited a small, non-statistically significant, reduced rotational behaviour compared to sham-operated rats. However, DI-MIAMI cells adhering to LM-coated PAMs resulted in a stabilization of the rotational behaviour at 2 weeks and a further constant, and statistically significant, decrease until the end of the experiment.

Protection of nigro-striatal pathway

The rotational behaviour was linked to the integrity of the lesioned nigro-striatal pathway, as observed using anti-TH immunohistochemistry (figure 18). Only a few TH positive fibres remained in the striatum of sham-treated rats 8 weeks after the lesion, suggesting that the retrograde neurodegeneration progressed in time concomitantly with the increased rotational behaviour. There was no major difference upon transplantation of DI-MIAMI cells alone, even if a small fraction of TH positive fibres were sometimes observed in the lesioned striatum compared to SHAM-treated rats.

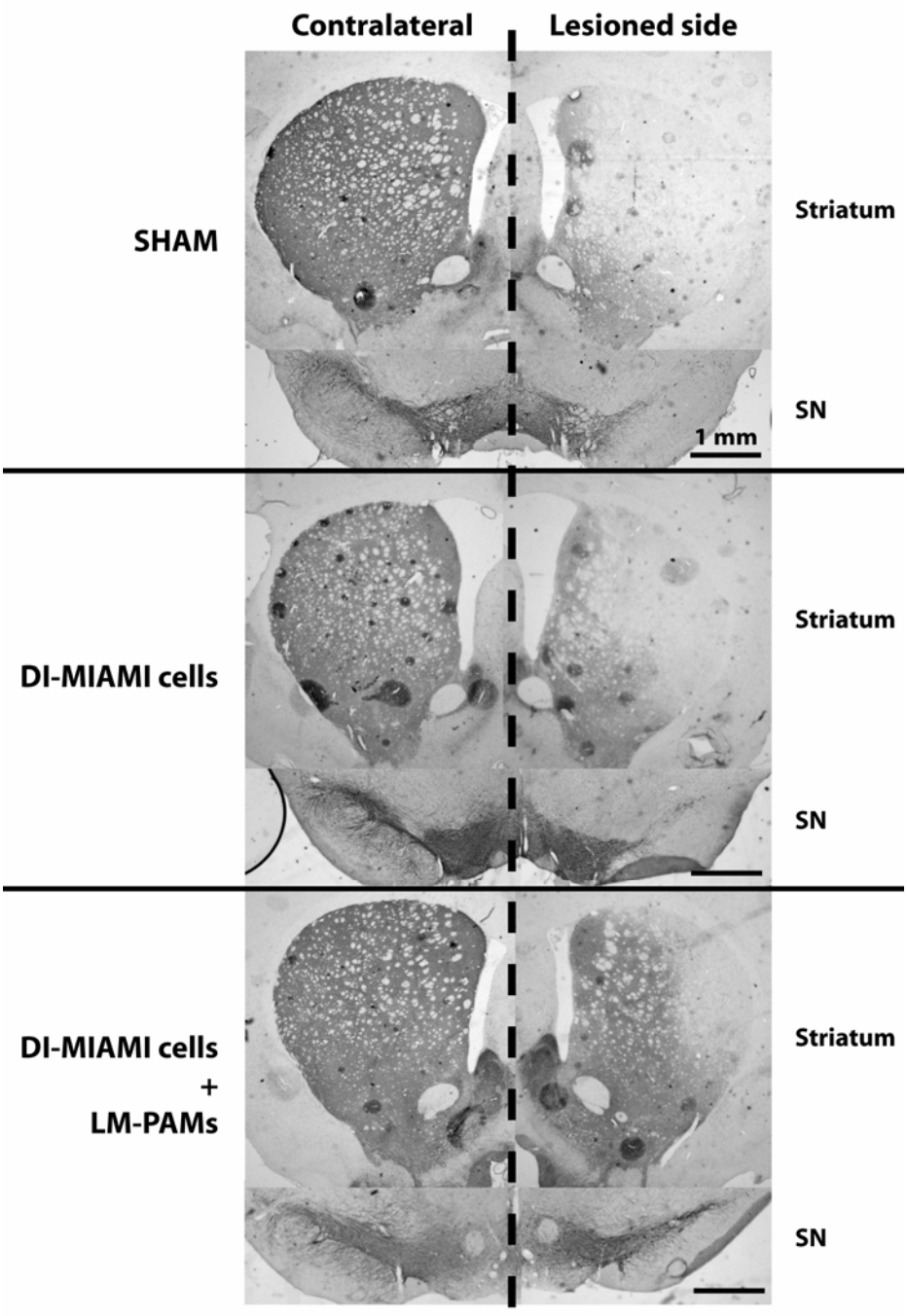

Figure 18. Neuroprotection of nigro-striatal pathway induced by LM-PAMs/DI-MIAMI cell complexes

Eight weeks after transplantation, only a few TH positive fibres were observed in the striatum as well as substantia nigra of rats receiving DI-MIAMI cells alone, while sham grafting resulted in an even more pronounced loss of these fibres. However, transplantation of DI-MIAMI cells in combination with LM-PAMs resulted in a more important neuroprotection of the dopaminergic fibres, as observed in the striatum and substantia nigra. Abbreviations: SN: substantia nigra, DI-MIAMI: dopaminergic-induced marrow-isolated adult lmultinlineage inducible, LM-PAMs: laminin-coated pharmacologically active microcarriers.

In both groups the number of neurons in the ipsilateral substantia nigra (SN) was importantly reduced compared to the contraletaral side. Transplantation of DI-MIAMI cells in combination with LM-PAMs resulted in a more important density of TH positive fibres in the striatum. In this case, a higher number of dopaminergic neurons were observed in the ipsilateral SN, suggesting that in this group of animals, a protection of the nigro-striatal pathway was mainly obtained rather than fibre outgrowth.

Cell fate in vivo

Despite the death of a consequent fraction of cells (approximately 80-90 %, with or w/o PAMs) observed by human genomic DNA quantification 2 weeks after transplantation, a significant number of cells were still detected 8 weeks after grafting in the striatum. Moreover, preliminary observations tend to demonstrate that the fraction of living cells may be higher after 8 weeks when combined with PAMs (figure 19). *In vitro*, the cytoplasmic membrane marker PKH26 did not diffuse to surrounding cells during coculture experiments (data not shown) and was consequently used to track transplanted DI-MIAMI cells *in vivo*. PKH26-positive cells co-localized nicely with the anti-human mitochondria-positive cells in the grafted area therefore confirming cell survival and integration within the parenchyma 2 months after transplantation. No strong inflammatory reaction was observed with OX42 (CD11b) staining and only a fraction of PKH26 dye colocalized with macrophage/microglia, suggesting their phagocytosis (figure 19).

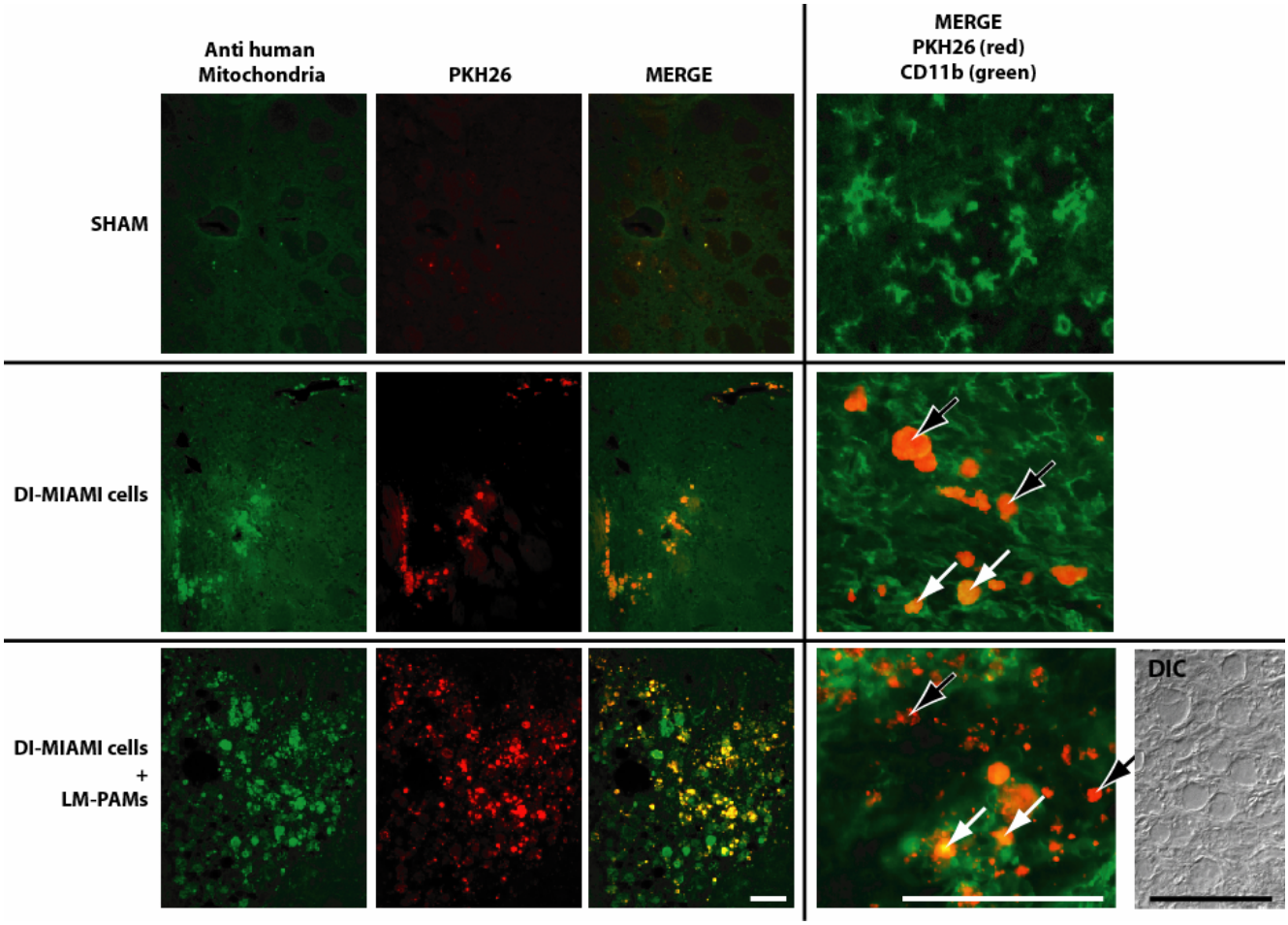

Figure 19. DI-MIAMI cells detection by immunohistochemistry 8 weeks after transplantation

DI-MIAMI cells were significantly detected in the striatum of rats, both with PKH26 and anti-human mitochondria staining which colocalized nicely. There was a possible increased survival of cells combined to PAMs compared to cells transplanted alone. Importantly, there was no strong immune reaction, and most of the PKH26 staining observed was not engulfed by macrophage/microglia (black arrows). White arrows point to engulfed PKH26. DIC: differential interference contrast image showing PAMs at the implantation site 8 weeks after grafting. Scale bars: 100 µm. Abbreviations: DI-MIAMI: dopaminergic-induced marrow-isolated adult lmultinlineage inducible, LM-PAMs: laminin-coated pharmacologically active microcarriers.

DISCUSSION

The potential of MSCs to treat neurodegenerative disorders, and especially PD is being investigated. However, the percentage of MSCs that survive and express neural/neuronal markers after transplantation in the brain is very low (Levy, Bahat-Stroomza et al. 2008; Delcroix, Jacquart et al. 2009). Functional improvements obtained in animal models of PD may primarily derive from the production of growth factors and chemokines by transplanted MSCs (Bouchez, Sensebe et al. 2008; Sadan, Bahat-Stromza et al. 2009). Tissue engineering aims at combining cells with a supportive element called a scaffold, with the ultimate goal of repairing lesioned tissues/organs. In this study, we combined a growth factor delivery vector developed in our laboratory, the PAMs, with DI-MIAMI cells to evaluate their potential protective/reparative effects on hemi-parkinsonian rats. The efficacy of this concept has been demonstrated in a PD animal model after intracerebral transplantation of NGF-releasing PAM conveying PC12 cells. These animals showed a behavioural improvement, and PC12 cell differentiation, reduced cell death and proliferation was observed (Tatard, Venier-Julienne et al. 2004). Similarly, GDNF-releasing PAM transporting embryonic dopaminergic cells induced an increased cell survival and dopaminergic fibre density resulting in behavioural improvement in hemiparkinsonian rats (Tatard, Sindji et al. 2007). After demonstrating the potential of LM to favour MIAMI cells neuronal differentiation *in vitro*, PAMs with a biomimetic surface of LM were formulated and further characterized. We here demonstrate the efficacy of this tool in a rat model of PD, as an important functional recovery was observed upon grafting with LM-PAMs/DI-MIAMI cells complexes, while no major effects were observed with DI-MIAMI cells alone. This behavioural recovery upon attachment of MIAMI cells to PAMs was correlated with neuroprotection of the nigro-striatal pathway, and was assumed to be due, at least partly, to the increased survival of cells which secrete a variety of neurotrophic factors and chemokines. As reported earlier, the PAMs may also improve cell differentiation and integration within the parenchyma.

We first demonstrated by RT-qPCR and flow cytometry that MIAMI cells expressed all the integrin receptors required for FN and LM binding (Docheva, Popov et al. 2007). Therefore, we analysed morphological and gene expression changes during *in vitro* neuronal differentiation on FN, LM or glass. MIAMI cells exhibited longer neurite-like extensions when differentiated on a substrate of LM *in vitro*, compared to FN or glass. This result was in accordance with a study that described morphological changes during *in vitro* neuronal differentiation of human MSCs on LM (Qian and Saltzman 2004). In the cited study, the

authors reported an increased percentage of human MSCs with secondary and tertiary branching during differentiation on LM compared to FN or PDL. Noteworthy, some studies describe conformational changes of LM, with varying biological effects, depending of the pH used during LM adsorption *in vitro*. In this sense, an acidic pH may be potentially advantageous in terms of neurite length and arrest of proliferation of rat embryonic cortical neurons (Freire, Gomes et al. 2002). In our hands, no significant changes were observed when differentiating MIAMI cells on a substrate of LM adsorbed at pH 4 or 7 so that we performed everything at neutral pH for convenience purposes. In addition to morphological changes, we also observed an improved expression of neural and neuronal proteins (β3-Tubulin and NFM, respectively) as well as a decreased, barely detectable, proliferation rate when MIAMI cells were differentiated on a LM substrate compared to FN. All these observations are commonly related to a normal stem cell differentiation process. Noteworthy, no cell body retraction was observed upon culture on a LM substrate, therefore confirming that the increased expression of β3-Tubulin and NFM were not artefacts due to cellular shrinkage as it has sometimes been suggested (Lu, Blesch et al. 2004; Khoo, Shen et al. 2008). Similarly, a study compared different surface chemistries and demonstrated that a substrate of LM induced Nestin expression by MSCs, a marker of neural precursors, prior to neuronal differentiation (Ho, Yu et al. 2006). All these results suggest a bioactive signalling role for LM in the induction of MIAMI cells toward a neuronal lineage.

PAMs with a biomimetic surface made of a combination of LM and PDL were used in this study. It was indeed compulsory to add PDL which is positively charged to allow an effective cell attachment to the PAM's surface. This resulted in a switch of the zeta potential of PAMs. PAMs coated with LM alone were negatively charged while PAMs coated using a mixture of LM/PDL were positively charged. As LM was used in combination with PDL, we confirmed that LM was homogeneously present all around the microspheres by IF and confocal microscopic observation. From a practical point of view, PAM's surface formulation was optimized, resulting in a shorter but also cheaper protocol as fewer proteins were used, without affecting surface characteristics in terms of zeta potential and homogeneity. Moreover, the reduced adsorption time (from 4 hours to 1.5 hours) may be especially advantageous as PAMs are also dedicated to encapsulate proteins, such as growth factors, which may be delivered *in vivo*. As a burst release is usually observed from PLGA microspheres during the first hours (Giteau, Venier-Julienne et al. 2008b), we may assume a significant amount of growth factor may be spared by reducing the adsorption time during surface formulation.

Cells attached to PAMs nicely promoted a reduction of the amphetamine-induced rotational behaviour, while cells alone did not induce a major recovery. This functional effect was correlated to the neuroprotection of the nigro-striatal pathway when DI-MIAMI cells were grafted in combination with LM-PAMs. Several mechanisms could be responsible for such an effect, the most important one being an improved cell survival upon attachment to PAMs, which may also result in an improved neuronal differentiation and further integration of DI-MIAMI cells within the brain. Furthermore, the production of growth factor and chemokines by these surviving cells may have protected the endogenous fibres from degenerating. Further investigations will be required to characterize such a production, both *in vitro* and *in vivo*. This mechanism is more likely to be responsible for the functional benefits observed, compared to a possible production of dopamine by *in situ* differentiated cells. Indeed, *in vitro*, dopaminergic induction for 48 hours did not result in major changes in the expression of *Nurr1, DAT* and *TH*, which were all detected at basal levels. However, the presence of TH positive DI-MIAMI cells *in vivo* still has to be addressed in more detail. We have previously shown that the dopaminergic inducing molecules used (SHH, FGF8b and retinoic acid) allowed a slight expression of Nurr1 after 48 hrs (step 2) and furthermore the expression of TH 3 days later, at the end of the induction protocol (step 3) (Tatard, D'Ippolito et al. 2007). In this study, the MIAMI cells were grafted at the end of step 2 suggesting that the dopaminergic phenotype was maybe not yet established. Noteworthy, a study described significant improvements obtained by grafting dopaminergic-induced human MSCs in the same animal model of Parkinson's disease (Bouchez, Sensebe et al. 2008). These authors also observed an incomplete engagement of MSCs toward the dopaminergic neuronal lineage and concluded that the functional effects observed derived from secretion of growth factors, chemokines or cytokines from the transplanted cells, a property the authors previously observed *in vitro* (Sensebe, Deschaseaux et al. 1997). Conversely, in our study, no major effects were observed when grafting DI-MIAMI cells without PAMs and we may assume these cells did not induce the appropriate paracrine effect, lost their differentiation potential *in vivo* or were more fragile after exposure to the dopaminergic induction protocol.

Interestingly, another team reported the efficient differentiation of hMSCs toward the dopaminergic phenotype *in vitro*, with TH expression and DOPA production (Levy, Bahat-Stroomza et al. 2008). Moreover, these dopaminergic-induced cells led to better recovery compared to naïve hMSCs, and a small fraction of cells exhibited dopaminergic characteristics *in vivo*, including TH expression. In their study, Levy et al used a total lesion model that resulted in a totally dopaminergic-deafferented striatum. As a consequence, the

improvements they observed were more likely to originate, at least partly, from MSCs dopaminergic neuronal differentiation and from a possible secretion of dopamine *in situ*. This was not likely to be the case in our study where a neuroprotection/neurorepair mechanism based on the secretion of growth factors by MSCs may preferentially have taken place.

In addition to the biomimetic approach, another way to improve the efficiency of cell grafts is to engineer a scaffold that may deliver a growth factor in a controlled fashion, further affecting the fate of both transplanted and host cells (see for review (Tatard, Menei et al. 2005; Orive, Anitua et al. 2009)). Indeed, synergistic effects between adhesion and growth factor signals to guide and enhance cell differentiation have now been described (Nakajima, Ishimuro et al. 2007). Such effects were also observed on dorsal root ganglion (DRG) neurons which exhibited an increased neurite length when cultivated in presence of LM and NGF *in vitro*, a cooperation that may, at least in part, be mediated via the phosphorylation of the protein Src (Tucker, Rahimtula et al. 2005), one of the first protein of the focal adhesion site being phosphorylated upon attachment of integrins to ECM molecules (Tucker, Rahimtula et al. 2008). In preliminary western blotting experiments made with MIAMI cells cultivated on LM, no phosphorylation of Src was observed at the tyrosine 416, responsible for the stabilisation of the active conformation of the protein (Engen, Wales et al. 2008). Further experiments are underway to investigate its phosphorylation at tyrosine 216 upon LM exposure, as was observed with DRG neurons (Tucker, Rahimtula et al. 2005). In a similar manner, a team reported the differentiation of NSCs into dopaminergic cells *in vitro*, upon culture on a LM substrate and exposure of cells to a combination of bFGF and heparin. Moreover, dopaminergic neurons were also observed *in vivo* after transplantation in a rat model of PD when this pre-treatment was used for cell priming (Yiqun Yu 2007). As MIAMI cells are known to differentiate toward the neuronal lineage in a NT3 dependent manner (Tatard, D'Ippolito et al. 2007), NT3 may be a suitable candidate for a future encapsulation into PAMs. Moreover, NT3 has also been shown to increase β3-Tubulin expression by MSCs during *in vitro* neuronal differentiation (Padovan, Jahn et al. 2003), a result we also previously demonstrated with MIAMI cells (Tatard, D'Ippolito et al. 2007)(Delcroix, Curtis et al., under review). Therefore, such LM-coated PAMs releasing NT3 and transporting MIAMI cells may further improve the benefits obtained with the use of LM-coated PAMs. This tissue engineering strategy may ultimately be used as a tool to increase the efficiency of MSCs therapy of the brain. To our knowledge, this is the first adult cell therapy strategy combining the biomimetic strategy with the controlled delivery of a growth factor to treat PD (Delcroix, Schiller et al., under review).

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DISCLOSURE OF INTERESTS

There is no disclosure of interest in this publication.

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RESULTATS-DISCUSSION

La LM a permis d'augmenter la réponse des cellules MIAMI aux stimuli du protocole de différenciation neuronale *in vitro*, avec une plus forte expression de marqueurs neuraux et neuronaux (β3-Tubuline et Neurofilament moyen (NFM) respectivement) en fin de différenciation par rapport à un substrat de FN ou de verre. La prolifération des cellules en cours de différenciation était également nettement diminuée sur un substrat de LM par rapport aux autres substrats. Enfin, les cellules de morphologie neuronale étaient significativement plus longues sur un substrat de LM par rapport aux autres substrats.

La phosphorylation de la protéine Src pourrait être impliquée dans la voie de signalisation responsable des effets de la LM sur la différenciation des cellules MIAMI (Tucker, Rahimtula et al. 2005; Rankin, Guy et al. 2008). Il est donc intéressant de vérifier si la LM utilisée dans notre étude provoque effectivement une activation de voies de signalisation intracellulaire via les intégrines. Si Src est impliqué dans cette voie de signalisation, la culture des cellules sur un substrat de LM devrait engendrer une phosphorylation plus importante de cette protéine, observable par Western Blot, par rapport à un substrat contrôle (PDL ou plastique). Des expériences préliminaires ont permis de montrer que les cellules MIAMI expriment la protéine Src, mais en très faible quantité, et que la phosphorylation du site Tyr 416, responsable de la stabilisation de la forme active de la protéine (Engen, Wales et al. 2008), n'est pas détectée après exposition des cellules MIAMI pendant 24 heures à un substrat de LM (figure 20). Le substrat de LM pourrait aussi induire la phosphorylation du site Tyr 216 de la protéine Src (Tucker, Rahimtula et al. 2005), et cette étude est actuellement en cours.

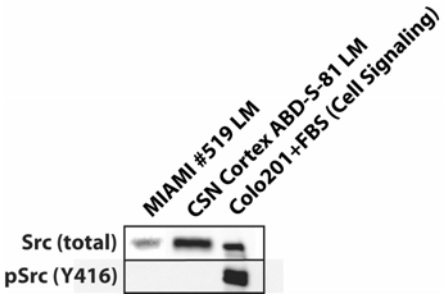

Figure 20. Western Blot de la protéine Src

Src est peu exprimée dans les cellules MIAMI (colonne 1). En revanche, des CSN humaines issues de cortex fœtal expriment fortement cette protéine (colonne 2). La phosphorylation de la tyrosine 416 n'est pas détectée après exposition des cellules MIAMI à un substrat de LM pendant 24 heures. Troisième colonne : contrôle positif pour la phosphorylation de la tyrosine 416 (20-30 µg de protéines totales/point).

Particulièrement intéressante, la protéine Src semble être à l'intersection des voies de signalisation induites par les molécules de la MEC et par les facteurs de croissances, tels le NGF, agissant en synergie pour induire la différenciation neuronale de cellules PC12 (Tucker, Rahimtula et al. 2005). Ainsi, l'étude de cette protéine pourrait offrir la possibilité de mettre en évidence une possible synergie entre l'effet de la LM recouvrant les MPA et le NT3 qui peut y être encapsulé, ces 2 facteurs favorisant la différenciation neuronale des cellules MIAMI. Cependant, il faut garder à l'esprit que d'autres voies de signalisation peuvent être impliquées dans ce mécanisme, l'inhibition de la voie des Rock/rho étant par exemple également connue pour son implication dans la différenciation neuronale de CSM (Pacary, Legros et al. 2006; Pacary, Petit et al. 2008).

Afin de bénéficier des avantages de la LM dans notre système *in vivo*, nous avons optimisé les MPA avec une surface biomimétique composée d'un mélange de LM et de PDL (MPA-LM). Tout en conservant un potentiel zêta largement positif, condition requise pour l'adhésion des cellules à la surface des microsphères, nous sommes parvenus à diminuer le temps d'adsorption des molécules de la MEC et de la PDL de 4h à 1h30. L'homogénéité de la surface a été vérifiée par immunofluorescence, bien que les quantités de LM utilisées aient été diminuées. Ces améliorations représentent un gain considérable en termes de temps et de coûts, mais de façon encore plus importante, la diminution du temps d'adsorption des molécules de la MEC limite les pertes de facteurs de croissance, comme le NT3, qui peuvent être encapsulés au sein des microsphères de PLGA. Cette surface biomimétique de LM et de PDL a permis une adhésion efficace des cellules MIAMI pré-traitées en EGF-bFGF et induites vers un phénotype dopaminergique. Il fut possible de faire adhérer 150000 cellules sur 0.75 mg de MPA pour nos transplantations intra-striatales.

Les transplantations de ces cellules combinées aux MPA-LM dans le modèle unilatéral et partiel de la maladie de Parkinson ont abouti à une nette récupération fonctionnelle, observée lors du test de rotation induite par les amphétamines, ainsi qu'en une protection importante du faisceau nigro-strié, alors que la greffe de cellules seules n'a pas induit d'effets majeurs. Ces résultats valident l'intérêt de cette approche d'ingénierie tissulaire pour améliorer les bénéfices de greffes de CSM pour le traitement de la maladie de Parkinson. Les mécanismes sous-jacents à cette amélioration fonctionnelle ainsi qu'à la réparation/protection des fibres dopaminergiques sont encore en cours d'études, mais doivent vraisemblablement être dus à une survie plus importante des cellules adhérées aux MPA. Ce phénomène peut également être lié à une possible amélioration de leur différenciation neuronale à leur contact. De plus, les MPA peuvent peut-être également modifier le profil de libération de facteurs de

croissances/chimiokines par les cellules MIAMI, aboutissant ainsi aux plus importants effets fonctionnels observés. Il est à noter que dans ce modèle de lésion partielle, la dégénérescence des neurones dopaminergiques endogènes a encore lieu au moment de l'implantation des CSM (2 semaines après lésion), de telle sorte qu'il est difficile de distinguer entre un éventuel mécanisme de réparation striatale, *via* une repousse des fibres dopaminergiques, et la protection contre la dégénérescence du faisceau nigro-strié. Idéalement, d'autres techniques de suivi non-invasives, telle l'imagerie en nanoSPECT ® (« single photon emission computed tomography ») ou micro-TEP (tomographie par émission de positons) pourraient permettre de répondre à cette question.

Des études de densité des fibres dopaminergiques au niveau du striatum nous permettront de quantifier plus précisément l'effet des greffes de MPA-LM/cellules MIAMI et plusieurs études sont actuellement en cours afin de valider les mécanismes à l'origine de la neuroprotection observée. La survie des cellules MIAMI, transplantées avec et sans MPA, pourra être quantifiée 8 semaines après transplantation à l'aide d'un marquage immunohistologique spécifique des cellules humaines. Une évaluation de la production de facteurs de croissance et de chimiokines, tels le « brain-derived neurotrophic factor » (BDNF), GDNF, NGF et le « vascular endothelial growth factor » (VEGF) (McCoy, Martinez et al. 2008), pourra également être évaluée *in vitro* avant et après adhésion sur les MPA par RT-qPCR. Enfin, des marqueurs neuraux et neuronaux nous permettront d'estimer la différenciation neuronale des cellules. Plus particulièrement, un anticorps polyclonal anti-TH, développé dans le cadre de cette étude et obtenu par immunisation de lapins à l'aide de 2 fragments peptidiques de la TH spécifiquement humaine (EDVRSPAGP & GTAAPAASYTPTPRS), devrait nous permettre d'évaluer la présence éventuelle de neurones dopaminergiques d'origine humaine, déjà décrite lors de la transplantation de CSM humaines dans un modèle de lésion totale de la maladie de Parkinson (Levy, Bahat-Stroomza et al. 2008). Notons que l'induction dopaminergique des cellules MIAMI, par exposition séquentielle au SHH et FGF8b puis à l'acide rétinoïque, semble avoir été incomplète vu la faible expression des gènes « dopaminergiques » tels *Nurr1*, *DAT* et *TH in vitro*. Nous avions cependant précédemment décrit une légère surexpression de Nurr1 à la fin de l'étape 2 de ce protocole de différenciation dopaminergique, et même de la TH suite à l'étape 3 (Tatard, D'Ippolito et al. 2007). Dans notre étude, les cellules ont été transplantées à la fin de l'étape 2 et la présence de cellules TH-positives, avec sécrétion éventuelle de dopamine, reste à confirmer *in vivo*.

Alors que la greffe de cellules MIAMI induites vers un phénotype dopaminergique n'entraîna pas d'effets majeurs sans MPA-LM, des expériences complémentaires nous ont permis de confirmer que les cellules MIAMI simplement pré-traitées en EGF-bFGF étaient capables à elles seules d'améliorer le comportement de rats hémi-parkinsoniens et d'induire une importante protection du faisceau nigro-strié (figure 21). Ces observations confirment l'effet thérapeutique des cellules MIAMI pré-traitées en EGF-bFGF dans ce modèle animal de la maladie de Parkinson, et confortent les effets observés lors de la greffe de CSM humaines, avec et sans induction dopaminergique, dans l'étude de Bouchez et al. (Bouchez, Sensebe et al. 2008). Il est notamment connu que les cellules différenciées, qui possèdent de plus grands prolongements que les cellules souches, sont plus sensibles au décollement ainsi qu'aux diverses étapes des protocoles de transplantations génératrices de stress cellulaire, ce qui pourrait être un élément de réponse expliquant la faible récupération observée avec les cellules induites vers un phénotype dopaminergique (Hermann, Gastl et al. 2004). De plus, ces cellules étant plus avancées dans le processus de différenciation possèdent peut-être un moindre potentiel de sécrétion de facteurs de croissance et de chimiokines que les cellules ayant seulement été pré-traitées en EGF-bFGF, ces dernières étant désormais connues pour leur forte production de facteurs (McCoy, Martinez et al. 2008).

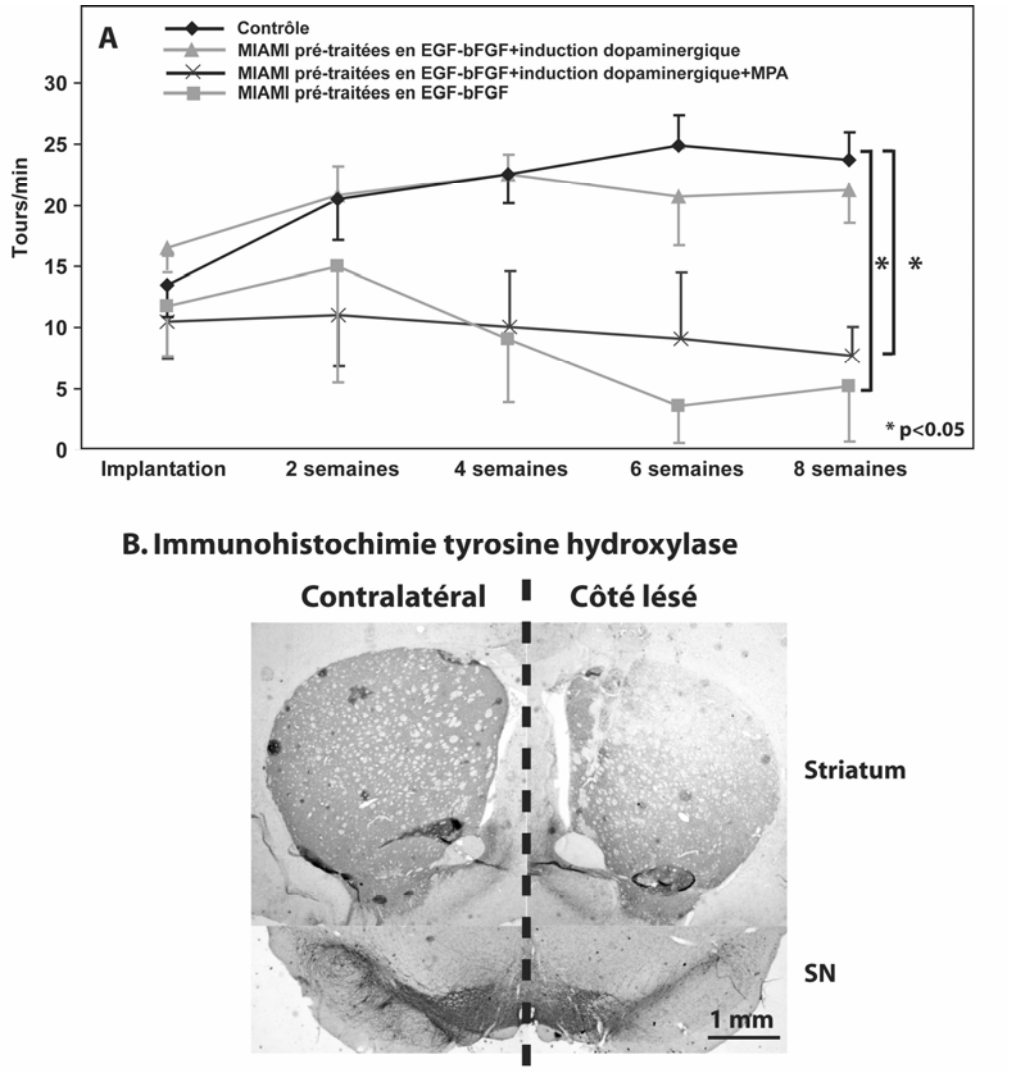

Figure 21. Comportement rotatoire induit par les amphétamines

Dans notre modèle de rats lésés à la 6-OHDA, la transplantation de cellules MIAMI pré-traitées en EGF-bFGF aboutit à une nette diminution du comportement rotatoire jusqu'à la fin du protocole expérimental, ce qui n'était pas le cas avec les mêmes cellules ayant subi une induction dopaminergique suite au pré-traitement (A). Cette induction dopaminergique ne causa également pas de changements significatifs d'expression des marqueurs dopaminergiques suivis. Les cellules MIAMI pré-traitées en EGF-bFGF, sans MPA étaient également capables d'induire une importante protection du faisceau nigro-strié (B).

Au vu des effets obtenus lors des greffes de cellules pré-traitées en EGF-bFGF présentées en figure 21, il semble intéressant de comparer dans le futur la survie entre ces cellules et celles ayant en plus subies une induction dopaminergique afin de déterminer le mécanisme à l'origine de cette différence de gain fonctionnel. Il serait également intéressant d'étudier l'effet des MPA-LM avec des cellules MIAMI se différenciant efficacement en cellules de phénotype dopaminergique, ainsi que l'effet des MPA sur les cellules MIAMI pré-traitées en EGF-bFGF, qui induisent déjà à elles seules un important gain fonctionnel et une

neuroprotection du faisceau nigro-strié (figure 21 et schéma récapitulatif des travaux en dernière page). Après validation de leur capacité de différenciation dopaminergique suite au pré-traitement en EGF-bFGF, les cellules MIAMI issues de la crête iliaque, qui possèdent un potentiel de différenciation neuronale similaire aux cellules MIAMI issues de vertèbres (chapitre 3), pourraient être utilisées avec les MPA-LM tout en autorisant un prélèvement aisé dans un contexte clinique. D'autres protocoles de différenciation vers un phénotype dopaminergique, tel un traitement favorisant l'augmentation du taux d'AMP cyclique en combinaison avec d'autres facteurs pourraient enfin être envisagés (Levy, Bahat-Stroomza et al. 2008).

Notre équipe a précédemment mis en évidence la dépendance des cellules MIAMI au NT3 au cours de leur différenciation neuronale *in vitro*, cette neurotrophine augmentant la survie et le pourcentage de cellules différenciées (Tatard, D'Ippolito et al. 2007). La formulation de microsphères de PLGA encapsulant le NT3 a été développée en utilisant le lysozyme comme protéine modèle afin d'obtenir une cinétique de libération optimale. D'ores et déjà, le NT3 a été précipité puis encapsulé avec succès au sein des microsphères de PLGA (~100 % d'encapsulation, estimé par quantification des protéines par fluorescence, NanoOrange Protein Quantitation Kit, Invitrogen). Pour cela, nous avons tout d'abord précipité la protéine dans des conditions salines favorables à ce phénomène ainsi qu'en la mettant en contact de glycofurol, un réactif provoquant la désolvatation du NT3. Les particules de protéines précipitées ont ensuite été resuspendues dans une phase organique contenant le PLGA, et le tout fut finalement émulsionné dans une phase aqueuse contenant une molécule tensioactive (alcool poly-vinylique) afin d'obtenir des microsphères de la taille désirée (60 µm) (figure 22).

Cette technique d'émulsion « solide/huile/eau », mise au point au sein de notre laboratoire (Giteau, Venier-Julienne et al. 2008b), permet de préserver l'intégrité de la protéine encapsulée en limitant ces interactions avec le polymère par rapport à une double émulsion « eau/huile/eau » plus classique, que nous utilisions auparavant. Les microsphères obtenues ont ensuite été recouvertes de LM et de PDL par simple adsorption à la surface du polymère et une cinétique de libération *in vitro* fut réalisée en plaçant les MPA dans un tampon de stabilité pour le NT3 (DPBS+0.1 % albumine bovine) à 37°C sous agitation.

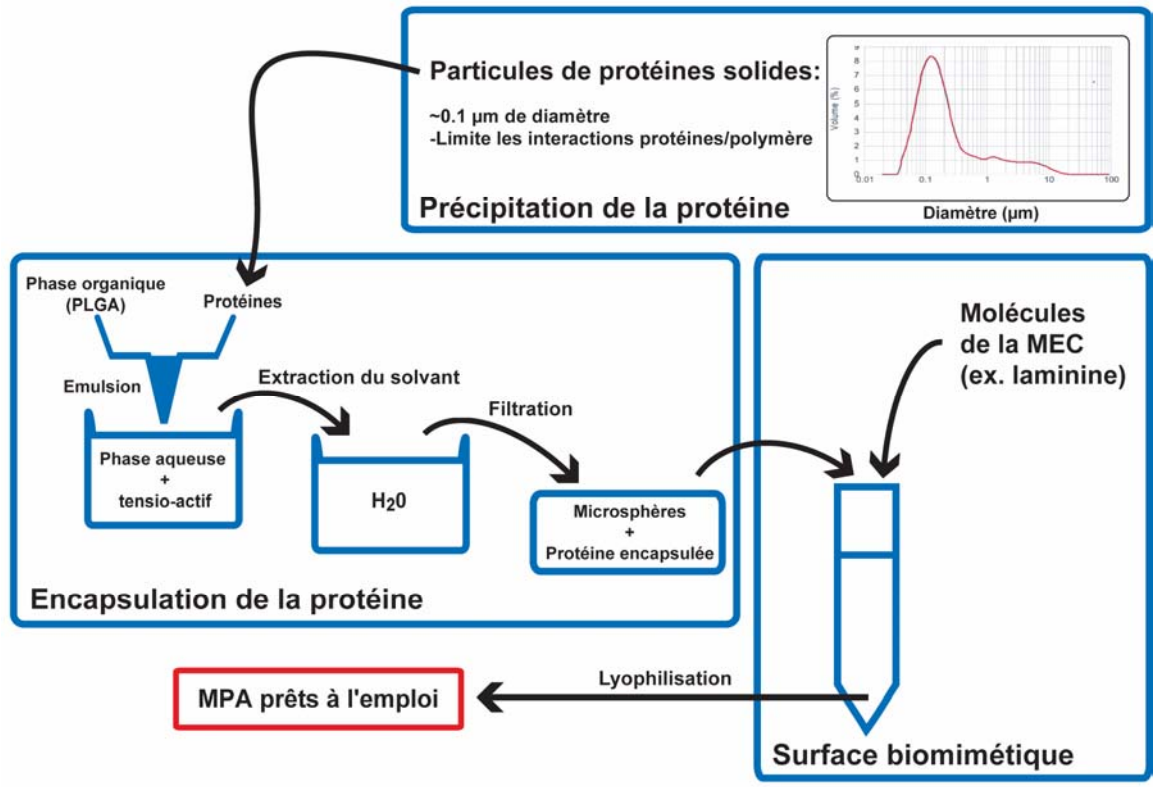

Figure 22. Etapes de formulation des MPA-NT3

La protéine d'intérêt (NT3) est encapsulée au sein des microsphères de PLGA par un procédé d'émulsion « solide/huile/eau » (Giteau, Venier-Julienne et al. 2008b). Les microsphères de 60 µm de diamètre obtenues sont ensuite recouvertes par une surface de molécules de la matrice extracellulaire (MEC) par adsorption, avant d'être lyophilisées pour conservation.

La technique « ELISA » s'est montrée inadéquate pour doser le NT3 libéré, en raison de son interaction avec l'un des composants de la formulation lors de l'étape de précipitation de la protéine. Cependant, un bioessai réalisé avec les cellules MIAMI a permis de confirmer que le NT3 encapsulé était toujours sous une forme biologiquement active. En effet, sans pré-traitement en EGF-bFGF et sur un substrat de FN, les cellules MIAMI prolifèrent une fois exposées au NT3 lors de l'étape d'engagement de notre protocole de différenciation *in vitro*. Cela nous a donc permis de doser approximativement le NT3 libéré par les MPA au cours du temps par quantification du nombre de cellules à l'aide d'un kit de dosage par fluorescence (Cyquant® cell proliferation assay kit, Invitrogen). Ainsi, environ 50 % du NT3 théoriquement encapsulé au sein des MPA était libéré et biologiquement actif après 22 jours de cinétique. Au vu de la cinétique présentée en figure 23, on peut donc supposer que 0.75 mg de MPA-LM-NT3 libèrent approximativement 75 ng de NT3 chaque jour durant les 3 premiers jours de libération, dose suffisante pour procurer d'éventuels effets thérapeutiques.

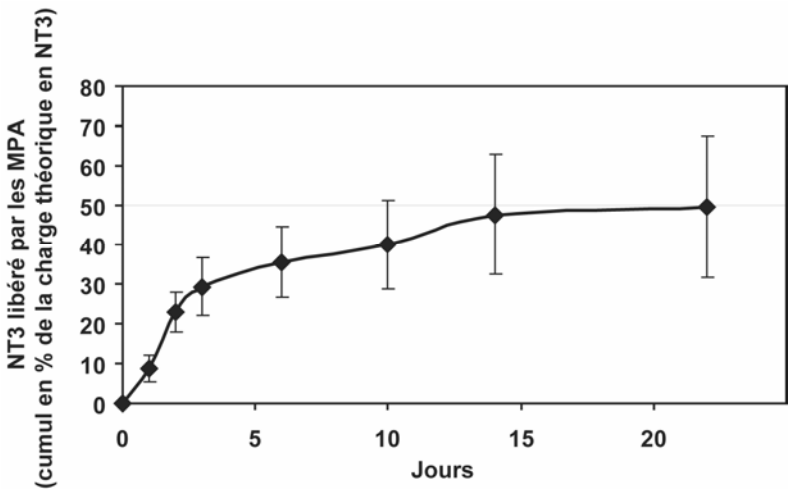

Figure 23. Cinétique de libération du NT3 *in vitro*

Les MPA ont été suspendus dans du tampon non dénaturant pour le NT3 et placés à 37°C dans un bain-marie sous-agitation. A plusieurs intervalles de temps, le tampon était prélevé puis congelé. A la fin de la cinétique de libération, tous les échantillons ont été présentés aux cellules MIAMI *in vitro*. L'augmentation de la prolifération observée fut ensuite comparée à une gamme étalon de cellules exposées à des concentrations connues de NT3 afin de déterminer les quantités de NT3 libérées aux différents temps. La cinétique de libération cumulée fait apparaître un pic de libération important durant les 3 premiers jours, avec un plateau vers 22 jours où environ 50 % du NT3 était libéré (résultats préliminaires, n=1).

Les greffes de cellules MIAMI, pré-traitées en EGF-bFGF, induites vers un phénotype dopaminergique et combinées à des MPA-LM-NT3 sont actuellement en cours. Des MPA contenant du NT3 ont également été implantés sans cellules, le NT3 libéré pouvant à lui seul avoir un effet bénéfique (neuroprotection/repousse des fibres) sur le parenchyme cérébral lésé. Comme précédemment, 0.75 mg de MPA ont été implantés avec $1.5x10^5$ cellules, ce qui représente une charge totale théorique de 0.75 µg de NT3 par greffe. Des résultats préliminaires semblent d'ores et déjà indiquer que les MPA-LM-NT3 seuls n'engendrent pas de récupération (fonctionnelle et histologique) majeure, contrairement aux MPA-LM-NT3 transportant les cellules MIAMI pré-traitées en EGF-bFGF et induites vers un phénotype dopaminergique.

DISCUSSION GENERALE ET PERSPECTIVES

Bien que de nombreuses études démontrent avec succès l'intérêt des CSM pour la thérapie cellulaire des os, du cartilage et des tendons, mais également d'organes tels le cœur et le foie (voir par exemple (Kharaziha, Hellstrom et al. 2009; Lasala and Minguell 2009; Quevedo, Hatzistergos et al. 2009; Sensebe, Krampera et al. 2009), un important fossé existe toujours entre les recherches de laboratoires, aussi fructueuses soient-elles, et le transfert de ces technologies vers la clinique. En effet, le changement d'échelle de l'animal à l'Homme cause des problèmes de coûts et de temps rendant le plus souvent difficilement réalisable l'application de ces protocoles à une plus grande échelle. Enfin, des critères de sécurité supplémentaires vis-à-vis des cellules et produits utilisés, dictés par les «current Good Manufacturing Practices » (cGMP) (Sensebe, Krampera et al. 2009), limitent également le nombre de recherches pouvant faire l'objet de transferts directs vers l'Homme. Cela explique, en partie, qu'il y ait actuellement encore peu d'études cliniques faisant appel aux cellules souches et à l'ingénierie tissulaire dans le contexte du cerveau (chapitre 1). Ainsi, il apparaît indispensable de mettre en place le plus tôt possible des protocoles utilisant au maximum des produits « sûrs », d'origine non-animale, et de préférence autorisés par les associations telles l'Agence Française de Sécurité Sanitaire des Produits de Santé (AFFSAPS) ou la FDA aux Etats-Unis. Les stratégies utilisées seront également avantageusement simples et peu onéreuses, afin d'anticiper un éventuel transfert vers la clinique.

Ce travail de thèse ayant pour objectif à très long terme d'implémenter une stratégie d'ingénierie tissulaire du cerveau chez l'Homme, nous nous sommes efforcés, dans la limite du réalisable à l'échelle du laboratoire, d'adopter des stratégies pouvant permettre une éventuelle transposition vers la clinique. Il nous a donc fallu tout d'abord connaître précisément le devenir après transplantation dans le cerveau des cellules choisies pour ces essais, ce qui constitue un véritable défi technologique. Pour cela, nous avons opté pour une technique de marquage permettant un suivi non-invasif des cellules par IRM et qui pourrait également être utilisée en clinique dans le futur.

Nous avons ainsi caractérisé le devenir de CSM transplantées dans le cerveau de rats après marquage avec des nanoparticules d'oxyde de fer enrobées de bisphosphonates (chapitre 2). Après en avoir validé l'innocuité sur les cellules, nous avons également validé la conservation du potentiel de différenciation ostéogénique et neurogénique des CSM après absorption des nanoparticules. Cette méthode nous a permis de mettre en évidence l'absence de migration des CSM transplantées dans un environnement cérébral sain, alors qu'une importante migration était observée vers le bulbe olfactif lorsque celui-ci était lésé (le récapitulatif des travaux de thèse est présenté en dernière page). Le suivi de cellules

transplantées *in vivo* est une problématique récurrente en biologie. En effet, quel que soit le marqueur utilisé, des doutes peuvent toujours subsister quant à son devenir en cas de division ou de mort des cellules transplantées, ou tout simplement en cas de « relargage » par celles-ci. Dans notre étude, si 90 % des CSM étaient marquées par les nanoparticules après la phase d'absorption, seulement 50 % des CSM restaient marquées après 3 jours de culture *in vitro*, ce qui peut éventuellement expliquer la faible fraction de cellules observées par bleu de Prusse *in vivo*. Ces problématiques peuvent se rencontrer avec la plupart des marqueurs utilisés expérimentalement pour suivre les cellules *in vivo*, comme le marqueur membranaire fluorescent PKH26, le marqueur d'ADN fluorescent Hoechst 33342, mais également les plus récents Quantum dots ®. Une étude a même récemment décrit un transfert possible vers les cellules hôtes du BrdU marquant l'ADN de cellules transplantées dans le cerveau (Burns, Ortiz-Gonzalez et al. 2006). Notons qu'au cours de nos travaux, nous avons observé la diffusion du Hoechst 33342 de cellules à cellules au cours d'expériences de co-culture *in vitro* alors que ce phénomène n'était pas observé avec le PKH26. Il est donc extrêmement difficile d'être certain que les cellules observées à l'aide de marqueurs soient effectivement les cellules transplantées et non pas, par exemple, des macrophages ou des neuroblastes endogènes ayant absorbé le marqueur utilisé. Une astuce technique pouvant permettre de s'assurer du devenir du marqueur utilisé consiste en l'injection de cellules marquées et préalablement lysées, et d'observer si le marqueur est observable dans les mêmes zones qu'en cas de transplantation de cellules marquées viables.

D'autres techniques de marquage ne sont quant à elles pas soumises à ces problèmes, comme des marquages immunohistologiques à l'aide d'anticorps spécifiques de l'espèce d'où sont issues les cellules transplantées, si celle-ci est différente de l'hôte. Il en est de même pour la technique « Fish », qui permet de suivre le devenir de cellules mâles transplantées dans un organisme de sexe opposé. Notons, que les allogreffes des cultures primaires de CSM réalisées dans cette première étude ne permettaient pas une implémentation aisée de ces techniques, contrairement aux xénogreffes de cellules MIAMI humaines et mâles réalisées par la suite. L'utilisation de cellules exprimant de façon constitutive un traceur observable en histologie est une autre alternative de suivi cellulaire. Ainsi, nous avons également tenté de marquer les cellules en les infectant par un lentivirus exprimant la « green fluorescent protein » (GFP). Cependant, les CSM GFP+ présentaient une importante diminution de leur prolifération ainsi que de leur potentiel de différenciation neuronale *in vitro*. A l'inverse des nanoparticules de fer, ce type de stratégies ne serait pas applicable en clinique à cause des risques liés à l'utilisation de vecteurs viraux. Certaines équipes ont également décrit

l'utilisation de cellules dérivées d'animaux transgéniques exprimant de façon constitutive la GFP, ce qui permettrait d'éviter les problèmes rencontrés lors de nos essais d'infection (Mothe, Kulbatski et al. 2005).

De manière générale, il apparaît important d'utiliser 2 marquages afin de s'assurer avec plus de confiance de la localisation des cellules transplantées. Ainsi, dans le chapitre 4, les nanoparticules d'oxyde de fer ont été utilisées en combinaison avec le BrdU dans le chapitre 2 et la visualisation du PKH26 a été couplée à un marquage immunologique anti-mitochondrie humaine.

La survie et la différenciation neuronale des CSM étaient particulièrement faibles dans cette première étude, raison pour laquelle nous nous sommes ensuite attachés à favoriser leur différenciation neuronale par un procédé simple consistant en un traitement en EGF-bFGF durant leur expansion (chapitre 3). Ce traitement a permis, à lui seul, de favoriser la spécification neuronale des CSM humaines MIAMI *in vitro*, résultant en une baisse de leur prolifération ainsi qu'en de nombreux changements d'expression de gènes, tous caractéristiques d'une spécification neuronale. De plus, ce traitement a également permis d'améliorer la réponse des cellules soumises au protocole de différenciation neuronale *in vitro*. Il est important de noter que ce protocole est essentiellement basé sur l'utilisation de facteurs de croissances. D'autres molécules sont néanmoins utilisées, principalement afin d'augmenter les taux d'AMP cyclique intracellulaire ou pour créer un environnement réducteur, les cellules neuronales étant particulièrement sensibles au stress oxydatif. En plus de leur caractère réducteur, le β-mercaptoethanol et le BHA, ont parfois été décriés pour un possible effet cytotoxique. Ainsi, il semble que des cellules d'aspect fibroblastique, comme les CSM, peuvent acquérir une morphologie neuronale suite à l'exposition à ces molécules, en raison d'une rétraction cytoplasmique (Neuhuber, Gallo et al. 2004; Bertani, Malatesta et al. 2005; Tao, Rao et al. 2005; Zurita, Bonilla et al. 2008). Cependant, dans notre étude, les changements morphologiques observés avaient lieu au cours du temps, durant les 3 étapes du protocole de différenciation, et ne peuvent pas être uniquement causés par un phénomène de rétraction cellulaire, comme cela a déjà été démontré dans d'autres études (Khoo, Shen et al. 2008). En effet, le cytoplasme des cellules MIAMI avant différenciation est déjà très peu étalé par rapport à des CSM cultivées de façon traditionnelle, et de très longues cellules de plus de 400 μm de long (~5 fois plus longues qu'en expansion) étaient observées en fin de différenciation. Des expériences de suivi vidéo de la formation des prolongements cellulaires pourraient permettre de confirmer encore davantage ce point. Enfin, des expériences préliminaires au cours desquelles le β-mercaptoethanol et le BHA ont été substitués par des

agents réducteurs plus acceptables d'un point de vue clinique (glutathion estérifié & α-tocotriénol), n'ont pas révélé de différences morphologiques significatives en fin de différenciation neuronale, en dépit d'une prolifération légèrement augmentée.

Les effets de ce traitement en EGF-bFGF et plus généralement tout résultat de ce type d'étude *in vitro* ne sont que des indicateurs du comportement possible des cellules *in vivo*, et doivent nécessairement être confortés par des études chez l'animal. Ainsi, soulignons que la transplantation de cellules pré-traitées en EGF-bFGF induit d'importants bénéfices fonctionnels dans un modèle de rats hémi-parkinsoniens (voir figure 21, page 160) ainsi que dans d'autres modèles animaux. Par exemple, dans un modèle d'ischémie cérébrale, une neuroprotection plus importante des neurones CA1 de l'hippocampe est observée après transplantation de cellules MIAMI pré-traitées en EGF-bFGF, par rapport à des cellules MIAMI cultivées de façon traditionnelle (projet en cours du Dr E. Garbayo). Cet effet peut être, tout au moins en partie, dû à la plus grande expression de facteurs de croissances et de chimiokines, comme cela a été démontré dans une population de CSM dérivée de tissus adipeux qui exprimait davantage de BDNF, GDNF, NGF et VEGF après traitement en EGF et bFGF (McCoy, Martinez et al. 2008). Ces cellules pré-traitées en EGF-bFGF étaient d'ailleurs capables d'induire une meilleure protection, et peut-être réparation, du faisceau nigro-strié que les mêmes cellules ayant en plus subi une induction dopaminergique (chapitre 4). Dans le cadre de la thérapie cellulaire du SNC, les effets bénéfiques des CSM semblent pouvoir provenir d'une véritable réparation cellulaire, en lien direct avec la capacité de différenciation des CSM, mais peut être de façon beaucoup plus importante de cette sécrétion d'une large variété de facteurs pouvant protéger le tissu hôte, induire sa reconstruction par les cellules endogènes ou encore moduler la réponse immunitaire (Bouchez, Sensebe et al. 2008; Horwitz and Prather 2009; Li and Chopp 2009), comme cela semble également le plus probable dans ce travail de thèse.

Il est important de préciser que ce pré-traitement en EGF-bFGF n'alourdit aucunement le protocole de culture des cellules, l'étape d'amplification étant nécessaire pour atteindre un nombre suffisant de cellules après isolation des CSM à partir de la moelle osseuse. Cependant, la culture des cellules MIAMI en faible densité rend notre protocole relativement coûteux et contraignant, celui-ci le devenant encore plus dans le cadre d'une éventuelle application humaine requérant un nombre beaucoup plus élevé de cellules. Des améliorations seraient ainsi nécessaires dans l'optique d'un possible transfert vers la clinique. Par exemple, quelques études ont décrit la culture de CSM en suspension (Hermann, Gastl et al. 2004; Kim, Honmou et al. 2006; Yang, Mu et al. 2008), ce qui a également déjà été réalisée avec les cellules

MIAMI au cours d'expériences préliminaires, permettant ainsi d'obtenir un grand nombre de cellules dans un plus petit volume, tout en limitant le stress induit aux cellules lors des traditionnels repiquages. L'utilisation de bioréacteurs rotatifs de type RCCS (« rotary cell culture system », Cellon, Bereldange, Luxembourg) semble notamment particulièrement adaptée à ce type de cultures limitant au maximum le stress cellulaire. Ces techniques de culture en système clos pourraient également permettre une automatisation de la culture des CSM, surmontant ainsi certaines difficultés posées par le transfert vers la clinique. Quelle que soit la méthode utilisée afin d'obtenir suffisamment de cellules pour une utilisation en clinique, il faudra de nouveau valider rigoureusement le potentiel thérapeutique des CSM obtenues par ces méthodes, avec un contrôle qualité adapté.

L'utilisation de sérum de veau fœtal (SVF) au cours de cette différenciation neuronale est également un point négatif pour une application clinique. Certains SVF ainsi que des sérums humains AB dits « sécurisés » sont désormais disponibles mais posent toutefois des problèmes de variabilité de lots à lots (Sensebe, Krampera et al. 2009). C'est la raison pour laquelle de nombreuses études s'attachent à optimiser la composition de milieux de culture supportant l'expansion ainsi que la différenciation des CSM sans sérum (Tao, Rao et al. 2005; Battula, Bareiss et al. 2007; Sensebe, Krampera et al. 2009).

Toujours dans le but d'améliorer le comportement des cellules après transplantation, à la fois en terme de survie et de différenciation, ce qui pourrait se traduire par une réparation cérébrale plus importante et peut-être par des effets plus prolongés, notre étude *in vivo* a fait appel à une approche d'ingénierie tissulaire utilisant les MPA en combinaison avec les cellules MIAMI, pré-traitées en EGF-bFGF et induites vers un phénotype dopaminergique. En effet, ayant démontré que la LM induisait une plus forte expression de marqueurs neuraux et neuronaux, ainsi qu'un changement de morphologie des cellules MIAMI *in vitro*, avec des cellules aux prolongements plus longs que sur d'autres substrats, nous avons développé des MPA munis d'une surface biomimétique de LM. Il est important de noter qu'une distance suffisamment faible entre cellules et MPA est requise pour que celles-ci soient en contact avec la surface biomimétique, mais également afin qu'elles soient sensibles au facteur libéré, ce dernier ayant une capacité de diffusion limitée dans le parenchyme cérébral (Tatard, Menei et al. 2005). La faible migration des CSM observée dans notre première étude, sauf en présence de lésion, suggère que les cellules restent majoritairement dans la zone lésée après transplantation dans le striatum de rats hémi-parkinsoniens, un comportement favorable à la réparation tissulaire.

Peu d'études décrivent l'utilisation de CSM en combinaison avec une approche d'ingénierie tissulaire pour la thérapie cellulaire du cerveau. Dans le cadre de la maladie de Parkinson, les MPA recouverts de cette surface de LM et transportant des cellules MIAMI induites vers un phénotype dopaminergique se sont montrés avantageux en termes de bénéfices fonctionnels et de protection du faisceau nigro-strié après transplantation dans le striatum de rats lésés à la 6-OHDA (chapitre 4). Ainsi, une baisse significative du nombre de rotations induites par les amphétamines était observée lorsque les cellules étaient utilisées en combinaison avec les MPA, un mécanisme ayant probablement pour origine une meilleure survie des cellules, alors que les cellules seules ne modifiaient que très peu le comportement des rats greffés. A notre connaissance, cette étude est l'une des premières à combiner efficacement cellules souches adultes et ingénierie tissulaire pour la thérapie de cette maladie neuro-dégénérative dans un modèle animal.

Des expériences préliminaires nous ont permis d'étudier *in vitro* l'influence du BDNF et du NT3 sur la différenciation neuronale des cellules MIAMI, dans la perspective d'encapsuler l'un de ces facteurs au sein des MPA. Nous avons ainsi confirmé l'importance du NT3 pour la différenciation neuronale des cellules MIAMI en terme d'expression de la β3-Tubuline et de NFM, effet déjà précédemment observé (Tatard, D'Ippolito et al. 2007), alors que l'effet du BDNF était moins significatif. Notons qu'il aurait été envisageable de co-encapsuler ces protéines si celles-ci s'étaient avérées d'égales importances, alors que d'autres techniques combinent microsphères et gels afin d'obtenir la libération des 2 protéines avec des cinétiques spécifiques (Burdick, Ward et al. 2006).

Par conséquent, nous avons choisi d'encapsuler le NT3 au sein de MPA recouverts de LM afin de favoriser l'effet de greffes de cellules MIAMI dans ce modèle de lésion partielle de la maladie de Parkinson. Comme décrit dans l'introduction de ce travail de thèse, la libération de protéines thérapeutiques de forte masse moléculaire sous une forme active est toujours un challenge technologique. On observe en effet, la plupart du temps, un pic de libération durant les premiers jours, puis un plateau après quelques semaines, sans parvenir à libérer la totalité de la protéine (voir figure 23, page 163). Une meilleure cinétique de libération (*i.e.* sans pic et plus complète) pourrait être obtenue avec l'utilisation de polymères plus hydrophiles, comme les polymères triblocs de PLGA-polyéthylène glycol (PEG)-PLGA (travaux de T. Van Tran & du Dr M.-C. Venier). Cependant, des études préliminaires d'immunofluorescence ont démontré une mauvaise adsorption des protéines de la MEC (FN et LM) sur ce polymère lors de la formulation des MPA, ce qui n'était pas le cas avec le PLGA traditionnel. D'autres techniques devront donc être étudiées afin de rendre possible

l'enrobage de microsphères de PLGA-PEG-PLGA par des molécules de la MEC. Une étude intéressante décrit notamment un traitement de surface pour des microsphères de PLGA avant adsorption de FN (Bible, Chau et al. 2009). Cependant, s'il s'avère réalisable dans le cas des MPA, ce type de traitement de surface ne devra ni dégrader la protéine encapsulée, ni interférer avec sa libération.

La preuve du concept des effets de la LM sur les CSM a été apportée dans le chapitre 4, mais notons que l'origine de cette protéine (placenta humain) interdit son utilisation en clinique. Cependant, grâce au nombre croissant d'études décrivant les effets de fragments synthétiques de la LM (chapitre 1), l'utilisation de cette stratégie nécessitera d'autres mises au point mais reste envisageable. Dans la même optique, il sera possible d'améliorer la formulation des MPA (voir figure 22, page 162) afin d'éviter l'utilisation de solvants organiques, détectables sous forme de traces dans le produit fini. Des méthodes dites de « prilling » (Berkland, Kim et al. 2001), une thématique actuellement développée au laboratoire (T. Van Tran et Dr M.-C. Venier), peuvent permettre d'encapsuler des protéines thérapeutiques sans solvant au sein de microsphères de PLGA, tout en permettant leur production à plus grande échelle. Cette méthode n'est pas basée sur un procédé d'émulsion et l'utilisation d'une phase organique n'est plus nécessaire. Le « prilling » est effectivement basé sur la solubilisation du polymère dans le glycofurol, dans lequel la protéine est également précipitée. Un flux de cette solution de polymère-protéines est ensuite dissocié en fines gouttelettes sous l'action d'ondes émises par un générateur d'ultra-sons, avant solidification dans un grand volume d'eau.

D'autres types de cellules souches, telles les cellules embryonnaires (ES), sont de meilleurs candidats en termes de potentiel de différenciation neuronale, à la fois *in vitro* et *in vivo,* et pourraient ainsi remplacer les cellules lésées dans le système nerveux central. De plus, on peut s'attendre à ce que cette capacité de différenciation neuronale procure un bénéfice en termes de survie cellulaire à plus long terme. Les cellules ES, en dépit des problèmes éthiques liés à leur origine, possèdent en effet des capacités de prolifération et de différenciation en neurones dopaminergiques matures jamais observées avec d'autres types de cellules souches, notamment par leur capacité à générer des potentiels d'actions et à former des synapses. A l'heure actuelle, les cellules ES sont effectivement encore les cellules pluripotentes par excellence, les autres sources de cellules souches, fœtales ou adultes, ne répondant habituellement pas aux critères de formation d'organismes chimériques après insertion dans un blastocyte. De nombreux protocoles permettant d'obtenir des neurones dopaminergiques ont été décrits, principalement par culture sur un lit de cellules nourricières (Laguna Goya,

Tyers et al. 2008) alors que d'autres se tournent désormais vers des alternatives, telles que l'utilisation de combinaison de facteurs, offrant potentiellement une plus grande reproductibilité et moins de problèmes d'applications en clinique pour la thérapie de la maladie de Parkinson (Vazin, Becker et al. 2009). Cependant une prolifération non-contrôlée des cellules ES après transplantation est parfois décrite (Aubry, Peschanski et al. 2009), et il est également peu aisé, d'un point de vue pratique, de cultiver des cellules ES en grande quantité.

Une autre source de cellules pluripotentes, les cellules « induced pluripotent stem cells » (iPS), ont été décrites en 2007 par Yamanaka et al. ainsi que par Thomson et al. (Takahashi, Okita et al. 2007; Yu, Vodyanik et al. 2007), et pourraient potentiellement constituer une source inépuisable de cellules souches pour la thérapie cellulaire de tous les organes, y compris pour la thérapie cellulaire de la maladie de Parkinson (figure 24).

La technologie iPS permet en effet de restaurer un état pluripotent chez une cellule somatique adulte, comme des fibroblastes, par l'expression de facteurs de transcriptions (habituellement 4 facteurs parmis c-Myc, KLF4, LIN28, Nanog, Oct3/4 and Sox2 selon les protocoles) induite par l'incorporation de retro- ou lentivirus. Cette technique très prometteuse a pour objectif principal l'obtention de cellules avec un potentiel de différenciation similaire aux cellules ES, tout en contournant le problème éthique majeur concernant l'utilisation de cellules embryonnaires. Les cellules iPS pourraient également permettre la réalisation d'autogreffes. Plusieurs types cellulaires ont déjà été obtenus en différenciant des cellules iPS *in vitro*, y compris des cellules de types hRPE (Buchholz, Hikita et al. 2009; Osakada, Jin et al. 2009), utiles pour la thérapie cellulaire de maladies de l'œil mais également pour la thérapie de la maladie de Parkinson en raison de leur capacité à sécréter de la L-DOPA ainsi que de la dopamine (chapitre 1). Des fibroblastes reprogrammés ont d'ores et déjà prouvé leur efficacité dans un modèle de rats de la maladie de Parkinson après avoir subi au préalable une re-différenciation vers un phénotype neuronal dopaminergique (Wernig, Zhao et al. 2008), confirmant ainsi l'intérêt de ces cellules pour la thérapie cellulaire des maladies neurodégénératives. Il est intéressant de noter que cette technologie peut être appliquée à des cellules autres que les fibroblastes. Ainsi des CSN ne requièrent la transduction que d'un seul facteur, Oct4, pour devenir pluripotentes (Kim, Sebastiano et al. 2009).

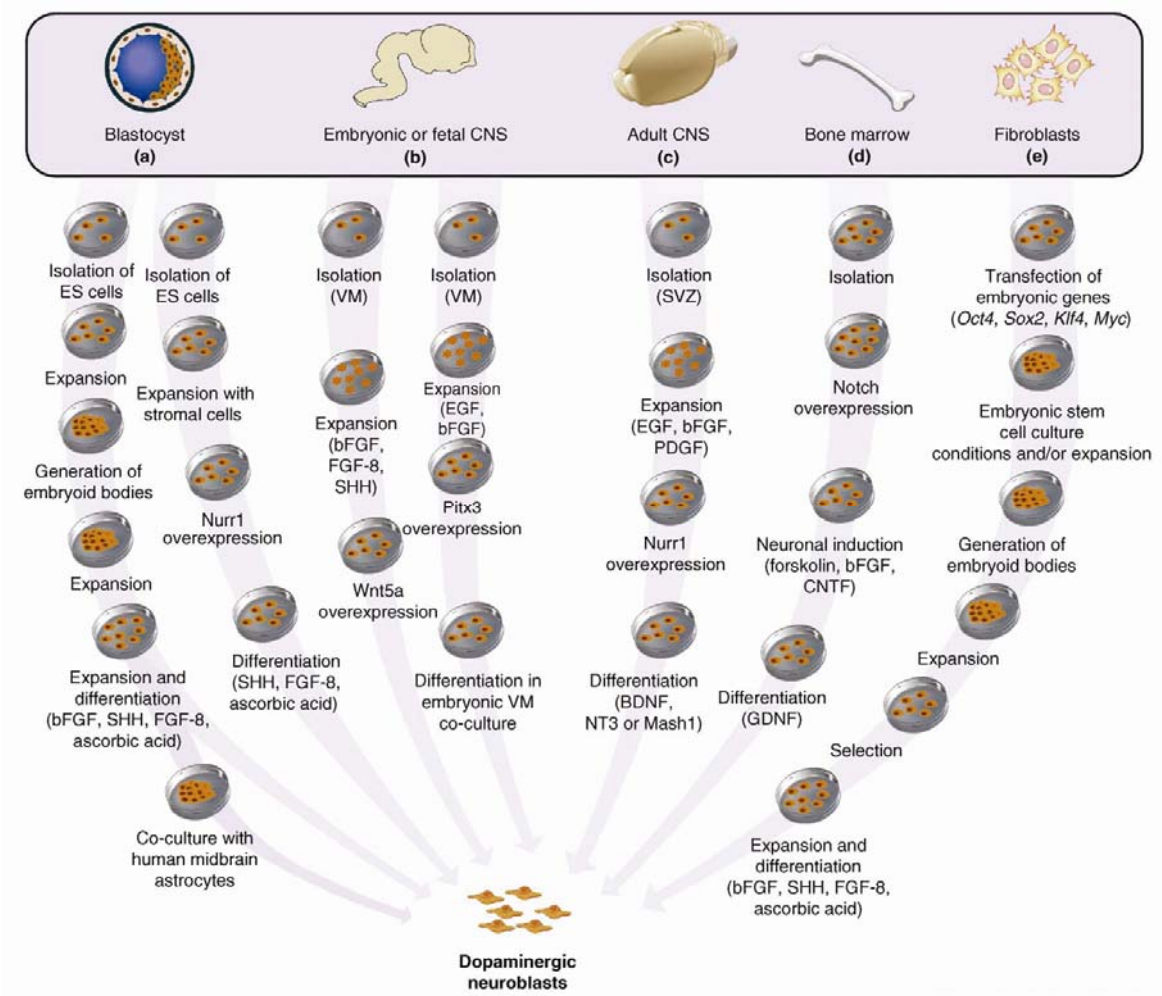

Figure 24. Récapitulatif des différentes sources possibles de cellules dopaminergiques pour la thérapie cellulaire de la maladie de Parkinson

Soulignons la possibilité de reprogrammer des cellules adultes, par exemple des fibroblastes, par transfection de facteurs de transcriptions. Ces cellules, appelées « induced pluripotent stem cells » (iPS) pourraient à terme devenir une source de cellules permettant la réalisation de greffes autologues pour la thérapie cellulaire de nombreux organes, y compris le cerveau dans le cadre de la maladie de Parkinson (figure reproduite de Lindvall et Kokaia, Cell, 2009).

Cette technologie fait actuellement l'objet d'intenses recherches afin d'éviter l'utilisation de vecteurs d'origines virales ainsi qu'une intégration génomique permanente qui restent des problèmes majeurs à surmonter avant d'éventuelles applications cliniques. En effet, ces techniques peuvent être à l'origine de mutations, de la formation de tumeurs ainsi que de difficultés pour différencier ces cellules vers un phénotype donné (Yu, Hu et al. 2009). De plus, la sûreté d'utilisation des cellules iPS semble variable selon le tissu d'origine, avec une plus ou moins forte propension à former des tératomes (Miura, Okada et al. 2009).

Néanmoins, divers travaux semblent indiquer qu'il soit possible d'abolir l'utilisation des vecteurs viraux, par exemple via l'application directe de protéines recombinantes (Gonzalez, Barragan Monasterio et al. 2009; Lee, Park et al. 2009; Page, Ambady et al. 2009; Yu, Hu et al. 2009; Zhou, Wu et al. 2009). Ainsi, il est possible que les cellules iPS deviennent effectivement une source de cellules pluripotentes de premier choix pour la thérapie cellulaire dans un futur proche.

Enfin, les possibilités offertes par l'ingénierie tissulaire afin de contrôler le « destin cellulaire » *in vivo* auront encore certainement un rôle à jouer afin de résoudre les problématiques essentielles de survie, d'absence de tératomes ainsi que de contrôle de la différenciation. Ainsi, si les MPA sont actuellement étudiés avec les CSM pour la thérapie cellulaire dans le contexte de maladies neurodégénératives, d'ischémies cérébrales mais également pour la réparation tendineuse et les ischémies cardiaques (avec des MPA encapsulant du « transforming growth factor-β3 » (TGF-β3) et du VEGF, respectivement), il est possible que cette stratégie d'ingénierie tissulaire soit avantageusement applicable aux cellules iPS dans le futur.

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CURRICULUM VITAE

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FORMATION

Depuis 2006-Doctorat en neurosciences-INSERM U646, Angers, France
Thèse réalisée sous la direction du Dr Claudia Montero-Menei, en collaboration avec une équipe de Miami, Floride

2006-Ingénieur UTC, génie biologique, filière conception et innovation de bioproduits
Université de Technologie de Compiègne (UTC), Compiègne, France
Dont 1 an en programme d'échange en Corée du Sud : Département « Chemical and Biomolecular Engineering » et laboratoire « Biosystem Engineering » du « Korean Advanced Institute of Science and Technology » (KAIST), Daejeon

2003-Diplôme d'Etudes Universitaires de Technologie (DEUTEC)-UTC
Dont 6 mois en programme d'échange en Irlande : «Galway Mayo Institute of Technology » (GMIT), Galway

2001-Baccalauréat scientifique, physique-chimie-Lycée François Bazin, Charleville-Mézières, France

FORMATION COMPLEMENTAIRES

2009-Diplôme d'expérimentation animale, niveau 1-Ecole Nationale Vétérinaire, Nantes, France

2007-Atelier de formation « Mécanismes moléculaires des signaux de transduction »-Braunschweig, Allemagne
Atelier proposé par le projet européen GENOSTEM

EXPERIENCES PROFESSIONNELLES

Depuis 2006-Doctorat en neurosciences-INSERM U646, Angers, France
Thèse intitulée « Cellules stromales mésenchymateuses et vecteurs polymériques pour l'ingénierie tissulaire du système nerveux central», visant à améliorer l'efficacité des transplantations de cellules souches, notamment dans le cadre de la maladie de Parkinson

-Définition et amélioration du potentiel de différenciation neuronale des cellules
(RT-qPCR, Western Blot, cytométrie en flux & immuno-marquage)
-Confirmation du potentiel de migration chez le rat par IRM, comparaison avec des cellules souches neurales
-Adaptation et caractérisation d'un vecteur polymérique servant de support aux cellules transplantées
(Mesures de tailles, microscopie électronique)
-Encapsulation d'une protéine (Neurotrophine 3) au sein de ce vecteur (microsphères de PLGA) & évaluation de la cinétique de libération (technique ELISA)
-Evaluation de l'homogénéité de l'enrobage de laminine, protéine de la matrice extra-cellulaire, de ce vecteur afin d'augmenter la survie et la différenciation des cellules in vivo (potentiel Zeta & immuno-marquage)
-Evaluation sur un modèle de rats hémi-parkinsoniens
(Stéréotaxie, RT-qPCR, histologie, microscopie à fluorescence & analyse d'images)
-Encadrement de stagiaires

Depuis 2008-Doctorat conseil-Université d'Angers, France
Mission de communication & de transfert de connaissances auprès d'entreprises privées
-Recherche de partenaires pour licence de brevet : prospection, rencontres avec industriels & congrès

2006 (6 mois)-Projet de fin d'études d'ingénieur-KAIST, laboratoire « Biosystem Engineering », Daejeon, Corée du Sud
Projet intitulé « Production de tréhalose par culture de mutants *Streptomyces lividans »,* ayant abouti à:
-La sélection et l'optimisation du milieu de culture favorisant la croissance des bactéries
-La quantification du tréhalose produit (HPLC & dosage enzymatique)

2005 (6 mois)-Stagiaire-FRIESLAND FOODS, laboratoire « Food Structuring », Deventer, Hollande
Projet ayant pour but la « Synthèse de nouvelles protéines de lait conjuguées à des oses » par réaction de Maillard
-Amélioration de la solubilité et de la stabilité thermique des protéines conjuguées
(Spectrophotométrie/turbidimétrie, dialyse, HPLC, calorimétrie & RMN à basse résolution)
-Augmentation de la stabilité thermique d'une solution de protéines de petit-lait après ajout de protéines conjuguées dans un ratio 1/10 (mesure de taille par diffraction, microscopie)

AUTRES EXPERIENCES

2009 (20 heures)-Encadrement de Travaux Pratiques d'immunologie-Université d'Angers, France
Supervision de groupes de 20 étudiants (2ème année de licence), enseignement des techniques d'immunologie couramment utilisées en laboratoire

2005 (1 an)-Responsable d'un Club Cuisine franco-coréen-Daejeon, Corée du Sud
Organisation logistique et animation des rencontres entre membres du « Cercle étudiant Chungnam National University (CNU)-KAIST », enseignement de recettes francophones aux étudiants coréens (~30 par séance)

2001-2004 (6 mois)-Ouvrier spécialisé-manutentionnaire-agent de maintenance
PLAFOMETAL, Monthermé, France
Production de plafonds métalliques et de tiges filetées, gestion du stock de matière première et distribution aux chaînes de production, maintenance du parc technique. Travail sur chaînes en horaires décalés (3-8)

1999-2001 (2 mois)-Manutentionnaire (pose de sièges de cinéma) & Magasinier de supermarché

RENSEIGNEMENTS COMPLEMENTAIRES

Langues : anglais courant
allemand, espagnol & coréen débutant

Ouverture à l'international : intégration facile dans des environnements professionnel et culturel différents, dans des pays étrangers ; travail quotidien avec des équipes non-francophones, rédaction de documents scientifiques en anglais (rapports, posters, publications scientifiques)

Informatique : Windows®, Word®, Excel®, Powerpoint®, Access®, Visual Basic®, Adobe photoshop®, Adobe illustrator®, Metamorph®

Intérêts : inter-culturalité, travaux manuels, gastronomie, sculpture, modélisme, course à pied, VTT & squash

Divers : Sauveteur Secouriste du Travail, permis B, avec véhicule

COMMUNICATIONS SCIENTIFIQUES

Publications internationales :

2009-Brain Research
Mesenchymal and neural stem cells labeled with HEDP-coated SPIO nanoparticles: *in vitro* characterization & migration potential in rat brain
***Gaëtan J.-R. Delcroix**, Matthieu Jacquart, Laurent Lemaire, Laurence Sindji, Florence Franconi, Jean-Jacques Le Jeune, Claudia N. Montero-Menei*

2007-Journal of microbiology and biotechnology
Expression and characterization of trehalose biosynthetic modules in the adjacent locus of the salbostatin gene cluster
*Choeng, Yong-Hoon, Ji-Yeon Yang, **Gaetan Delcroix**, Yoon Jung Kim, Yong Keun Chang, Soon-Kwang Hong*

Conférences :

2009-Journée du Service Commun d'Imageries et d'Analyses Microscopiques (SCIAM)
UFR Sciences médicales, Angers, France
Cellules souches mésenchymateuses et vecteurs polymèriques pour la thérapie cellulaire du système nerveux central
***G.J.-R. Delcroix**, M. Jacquart, O. Thomas, L. Sindji, L. Lemaire, C.N. Montero-Menei*

Posters :

2009-Congrès de l'« International Society for Stem Cell Research » (ISSCR)-Barcelone, Espagne
EGF-bFGF pre-treatment and laminin-coated Pharmacologically Active Microcarriers delivering Neurotrophin 3 to improve human stromal MIAMI cells survival and differentiation in a rat model of Parkinson's Disease
***G.J.-R. Delcroix,** K. Curtis, L. Sindji, J.-P. Karam, O. Thomas, J.-P. Benoit, P.C. Schiller, C.N. Montero-Menei*

2009-5ème colloque national des cellules souches IFR26-Nantes, France
Microcarriers pharmacologiquement actifs et cellules stromales humaines « MIAMI » pour la thérapie cellulaire du système nerveux central
***G.J.-R. Delcroix**, L. Sindji, O. Thomas, P. Schiller, C.N. Montero-Menei*

2009-Congrès Gen2bio-La Baule, France
Pharmacologically active microcarriers : application for cell therapy of the central nervous system with human stromal MIAMI cells
***G.J.-R. Delcroix**, O. Thomas, L. Sindji, J.P. Karam, J.P. Benoit, C.N. Montero-Menei*

2008-Congrès de l'« International Society for Cell Therapy » (ISCT)-Miami, USA
Pharmacologically Active Microcarriers : a tool to explore the pluripotentiality of human stromal MIAMI cells for cell therapy
***G.J.-R. Delcroix**, K. Curtis, P.C. Schiller, G. D'Ippolito, J.P. Benoit, C.N. Montero-Menei*

2007-Congrès du projet européen « GENOSTEM »-Montpellier, France
Pharmacologically active microcarriers : a tool to explore the pluripotentiality of MSC for cell therapy
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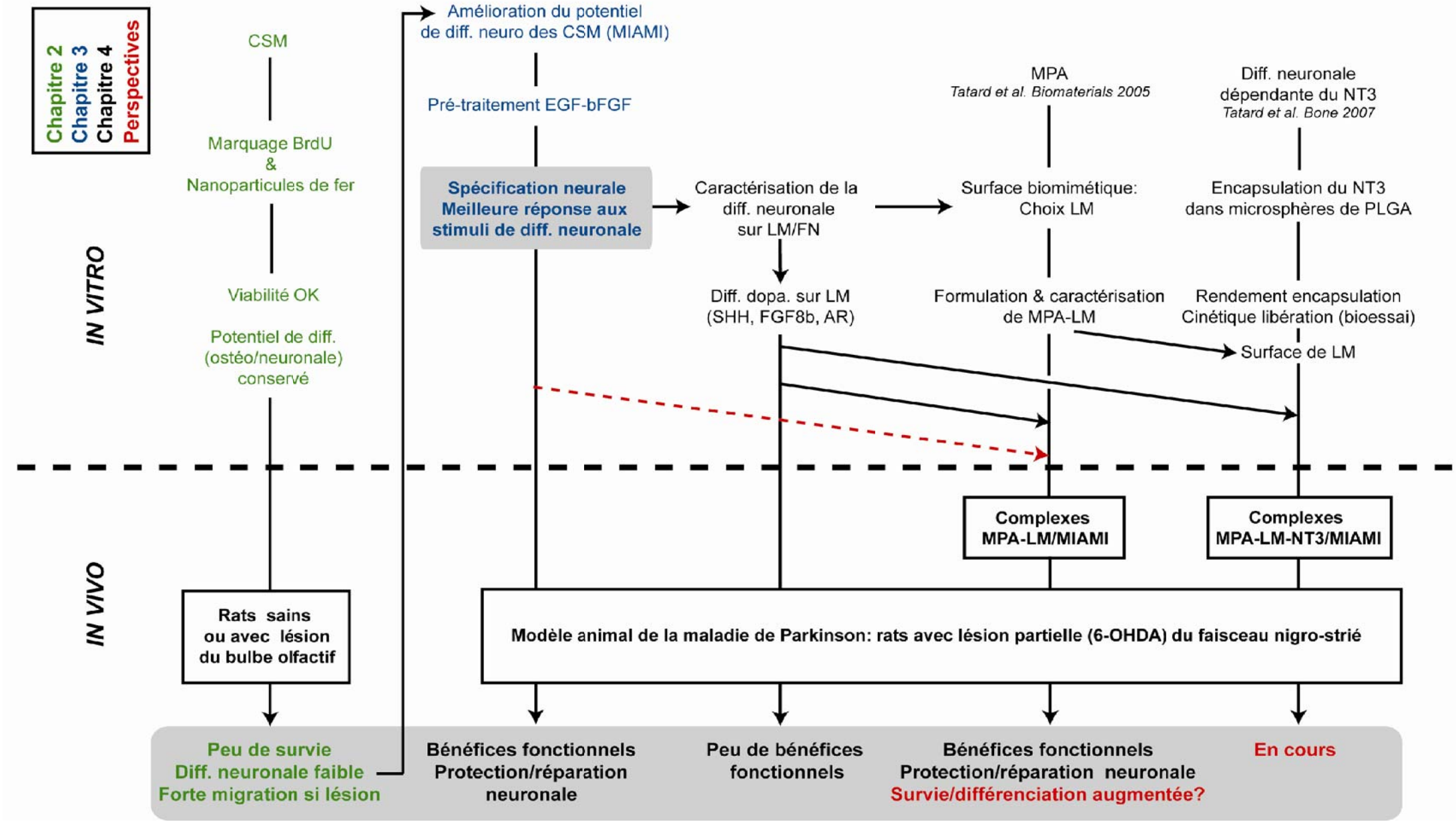
Chapitre 2
Chapitre 3
Chapitre 4
Perspectives
IN VITRO
IN VIVO
CSM
Marquage BrdU
&
Nanoparticules de fer
Viabilité OK
Potentiel de diff.
(ostéo/neuronale)
conservé
Amélioration du potentiel
de diff. neuro des CSM (MIAMI)
Pré-traitement EGF-bFGF
Spécification neurale
Meilleure réponse aux
stimuli de diff. neuronale
Caractérisation de la
diff. neuronale
sur LM/FN
Diff. dopa. sur LM
(SHH, FGF8b, AR)
MPA
Tatard et al. Biomaterials 2005
Surface biomimétique:
Choix LM
Formulation & caractérisation
de MPA-LM
Diff. neuronale
dépendante du NT3
Tatard et al. Bone 2007
Encapsulation du NT3
dans microsphères de PLGA
Rendement encapsulation
Cinétique libération (bioessai)
Surface de LM
Complexes
MPA-LM/MIAMI
Complexes
MPA-LM-NT3/MIAMI
Rats sains
ou avec lésion
du bulbe olfactif
Modèle animal de la maladie de Parkinson: rats avec lésion partielle (6-OHDA) du faisceau nigro-strié
Peu de survie
Diff. neuronale faible
Forte migration si lésion
Bénéfices fonctionnels
Protection/réparation
neuronale
Peu de bénéfices
fonctionnels
Bénéfices fonctionnels
Protection/réparation neuronale
Survie/différenciation augmentée?
En cours

RESUME

La thérapie cellulaire constitue une alternative prometteuse pour de nombreuses atteintes cérébrales, telles les maladies neurodégénératives, pour lesquelles aucun traitement réparateur n'est actuellement disponible. Les cellules stromales mésenchymateuses (CSM) adultes ne soulèvent pas de problèmes d'ordre éthique et permettent de réaliser des greffes autologues après prélèvement de moelle osseuse du patient, sélection, puis amplification des cellules *in vitro*. Seules ou associées à des biomatériaux dans le contexte de l'ingénierie tissulaire, ces cellules sont largement étudiées pour la régénération de nombreux tissus conjonctifs en vertu de leurs propriétés immuno-modulatrices, de leur capacité à produire des facteurs de croissance et chimiokines, mais également de leur large potentiel de différenciation. Plus récemment, les CSM font l'objet d'un nombre croissant d'études visant à la restauration des fonctions du système nerveux central endommagé, et plus particulièrement du cerveau.

Nous avons ainsi caractérisé le comportement de CSM après transplantation dans le cerveau de rats adultes, en termes de survie, de différenciation neuronale et de potentiel de migration. Une méthode de suivi basée sur l'incorporation de nanoparticules d'oxyde de fer nous a permis de montrer que les CSM ne migraient pas dans un cerveau sain alors qu'elles étaient attirées par une lésion située à grande distance de leur site d'implantation. Nous avons également confirmé que la faible survie et différenciation des cellules *in vivo* constituent les obstacles majeurs à la thérapie cellulaire du cerveau.

Par conséquent, nous nous sommes attachés à améliorer le potentiel de différenciation neuronal des CSM avant transplantation. Les CSM humaines MIAMI (marrow isolated adult multilineage inducible) sont capables de se différencier en cellules de phénotype neuronal, sous la dépendance de la neurotrophine-3 (NT3). Un pré-traitement en « epidermal growth factor » (EGF) et « basic fibroblast growth factor » (bFGF) durant l'expansion de ces cellules a permis d'orienter leur spécification vers un phénotype neuronal. De plus, ce pré-traitement a également induit une baisse de la prolifération en cours de différenciation *in vitro*, ainsi qu'une expression plus forte de marqueurs neuraux et neuronaux. Les cellules différenciées suite au pré-traitement présentaient un corps cellulaire fin avec de très longs prolongements; une morphologie comparable à celle de cellules neuronales.

L'ingénierie tissulaire pourrait permettre d'améliorer le comportement des cellules transplantées en les combinant avec des biomatériaux, dans le but de réparer un tissu lésé. Ainsi, nous avons associé ces cellules MIAMI à des vecteurs polymériques, les microcarriers pharmacologiquement actifs (MPA), afin de favoriser la survie, la différenciation neuronale et les capacités de réparation tissulaire des cellules MIAMI après transplantation. Ces microsphères de PLGA, biodégradables et biocompatibles, ont ainsi été enrobées de laminine (LM), une molécule de la matrice extracellulaire, après en avoir démontré les bénéfices sur la différenciation neuronale des cellules MIAMI *in vitro*. Des cellules MIAMI, prétraitées en EGF-bFGF et induites vers un phénotype dopaminergique, ont ensuite été mises en contact avec des MPA-LM libérant du NT3 avant transplantation dans un modèle animal de la maladie de Parkinson. Cette stratégie a permis de mettre en évidence d'importants effets fonctionnels par rapport à la greffe de cellules seules, jusqu'à 8 semaines après transplantation.

Cette stratégie d'ingénierie tissulaire est, à notre connaissance, la première à démontrer l'intérêt de cellules souches adultes associées à des vecteurs polymériques bioactifs pour protéger le système nerveux central dans le contexte de la maladie de Parkinson.

Mots clés : thérapie cellulaire, ingénierie tissulaire, cellules stromales mésenchymateuses, migration, EGF, bFGF, maladie de Parkinson, microcarriers pharmacologiquement actifs, laminine, fibronectine, neurotrophine 3

Thèse G. Delcroix

Titre francais :

Cellules stromales mésenchymateuses et vecteurs polymériques pour l'ingénierie tissulaire du système nerveux central.

Résumé français :

Les cellules stromales mésenchymateuses (CSM) possèdent de nombreux atouts pour la thérapie cellulaire du cerveau. Nous avons tout d'abord démontré que les CSM ne migraient pas dans le cerveau de rats sains alors qu'elles étaient attirées par une lésion située à grande distance de leur site d'implantation. Nous avons également confirmé que la faible survie et différenciation neuronale des cellules *in vivo* constituent les obstacles majeurs à la thérapie cellulaire du cerveau.

Par conséquent, nous nous sommes ensuite attachés à améliorer le potentiel de différenciation neuronal des CSM avant transplantation, à l'aide d'un pré-traitement en « epidermal growth factor » (EGF) et « basic fibroblast growth factor » (bFGF) *in vitro*.

Finalement, nous avons associé des CSM à des vecteurs polymériques, les microcarriers pharmacologiquement actifs (MPA), afin de favoriser la survie, la différenciation neuronale et les capacités de réparation tissulaire des cellules après transplantation. Ces microsphères de PLGA ont ainsi été enrobées d'une surface biomimétique de laminine, après en avoir démontré les bénéfices sur la différenciation neuronale des CSM *in vitro*. Des CSM ont ensuite été mises en contact avec des MPA enrobées de laminine et libérant une neurotrophine, avant transplantation dans un modèle animal de la maladie de Parkinson.

D'importants effets fonctionnels ont été observés par rapport à la greffe de cellules seules, et cette stratégie est la première à démontrer l'intérêt de cellules souches adultes associées à des vecteurs polymériques bioactifs pour protéger le système nerveux central dans le contexte de la maladie de Parkinson.

Mots clés :

Ingénierie tissulaire, cellules stromales mésenchymateuses, EGF-bFGF, maladie de Parkinson, microcarriers pharmacologiquement actifs

Titre anglais :

Mesenchymal stromal cells and polymeric vectors for tissue engineering of the central nervous system.

Résumé anglais :

Mesenchymal stromal cells (MSCs) have several advantages for brain cell therapy. We first demonstrated that MSCs do not migrate in healthy rat brains whereas they were attracted by a lesion located far from their implantation site. However, we also confirmed that the poor cell survival and neuronal differentiation are the major obstacles encountered for brain cell therapy.

Thus, we then focused on enhancing the neuronal differentiation potential of MSCs before transplantation, using an epidermal growth factor-basic fibroblast growth factor (EGF-bFGF) pre-treatment *in vitro*.

Finally, we associated MSCs to polymeric vectors, the pharmacologically active microcarriers (PAMs), to increase cell survival and neuronal differentiation, and thereby favouring MSC tissue regeneration potential after transplantation. These PLGA microspheres were coated with a biomimetic surface of laminin, after demonstrating the benefits of this molecule on the neuronal differentiation potential of MSCs *in vitro*. After attachment of MSCs on their surface, neurotrophin releasing, laminin-coated PAMs have been evaluated in a rat model of Parkinson's disease.

A significant functional recovery was observed compared to cells grafted without PAMs. This strategy is the first to give the proof of concept for the use of adult stem cells combined to bioactive polymeric vectors to protect the central nervous system in the context of Parkinson's disease.