Conception de biomatériaux hybrides à base de cellules souches pour l'ingénierie tissulaire

Benjamin Mesure

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Par **Benjamin MESURE**

Conception de biomatériaux hybrides à base de cellules souches pour l'ingénierie tissulaire

Soutenue le 28 septembre 2018

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-

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"Transforming growth factor-beta 1 or ascorbic acid are able to differentiate Wharton's jelly mesenchymal stem cells towards a smooth muscle phenotype."
Bio-Medical Materials and Engineering 2017

Dan P., Velot É., **Mesure B.**, Groshenry G., Bacharouche J., Decot V., Menu P.
"Human umbilical cord derived matrix: A scaffold suitable for tissue engineering application."
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Dostert G., **Mesure B.**, Menu P., Velot É.
"How Do Mesenchymal Stem Cells Influence or Are Influenced by Microenvironment through Extracellular Vesicles Communication?"
Frontiers in Cell and Developmental Biology 2017

Dostert G., Kahn C. J. F., Menu P., **Mesure B.**, Cleymand F., Linder M., Velot É., Arab-Tehrany E.
"Nanoliposomes of Marine Lecithin, a New Way to Deliver TGF-β1."
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"Influence of umbilical cord extracellular matrix on the chondrogenic potential of human Wharton's jelly mesenchymal stem cells genetically modified to overexpress SOX9 via rAAV-mediated gene transfer".
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B. Mesure, G. Dostert, V. Jouan-Hureaux, H. Louis, I. Motorine, P. Menu, V. Decot, É. Velot. "Evaluation of the CD73 transfer into NK cells by exosomes derived from mesenchymal stem cells and its immunomodulatory effect."
1ère Journée Pharma Recherche. Mars 2017 Nancy (France)

Liste des abréviations

AA : Acide ascorbique

ADN : Acide désoxyribonucléique

BMP : Bone morphogenic protein

CE : Cellule endothéliale

CML : Cellule musculaire lisse

CSM : Cellule souche mésenchymateuse

CSM-GW : Cellule souche mésenchymateuse de la gelée de Wharton

FGF : Fibroblast growth factor

HUVEC : Human umbilical vein endothelial cell

UMR IMoPA : Unité mixte de recherche Ingénierie Moléculaire et Physiopathologie Articulaire

MEC : Matrice extracellulaire

MEC-GW : Matrice extracellulaire de la gelée de Wharton

MEC-TA : Matrice extracellulaire de tissu adipeux

NO : Monoxyde d'azote

PDGF : Platelet-derived growth factor

rAAV : Adénovirus associé recombinant

RT-pPCR : Real time – quantitative polymerase chain reaction

SVF : Sérum de veau fœtal

TA : Tissu adipeux

TGF : Transforming growth factor

VEGF : Vascular endothelial growth factor

ZEO : Zentrum für Experimentelle Orthopädie

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Introduction générale

Les pathologies qui atteignent les os ou le cartilage affectent la majorité des personnes, qu'elles soient dues à des traumatismes ou à des maladies dégénératives. Il arrive que les traitements disponibles pour les traiter ne permettent pas une restauration complète du tissu. La médecine régénératrice, et plus précisément l'ingénierie tissulaire, cherche à répondre à ce manque en reproduisant les tissus perdus à partir de cellules souches et de biomatériaux, qui peuvent être extraits de tissus humains comme la gelée de Wharton, le tissu conjonctif du cordon ombilical. Les tissus osseux et cartilagineux ont chacun leurs propriétés et contraintes qui doivent être soigneusement prises en compte dans chaque entreprise d'ingénierie. Par exemple, le tissu osseux nécessite une vascularisation riche pour assurer pleinement sa fonction, tandis que le cartilage ne possède pas de vaisseaux.

Dans ces travaux de thèse, le but a été d'utiliser les propriétés des cellules souches mésenchymateuses de la gelée de Wharton pour les différencier vers des phénotypes vasculaires ou chondrocytaires, en les associant à la matrice extracellulaire de cette même gelée. Pour potentialiser les effets de ces cellules, des techniques de thérapie génique ont été employées pour induire la production de facteurs de différenciation par les cellules. Ainsi j'ai pu bénéficier de l'expertise du Zentrum für Experimentelle orthopädie dans la thérapie génique utilisée notamment pour traiter les pathologies cartilagineuses et osseuses, que j'ai associé aux techniques d'ingénierie utilisées dans le groupe « ingénierie vasculaire » d'IMoPA pour reconstruire les tissus vasculaires et articulaires. Ceci dans le but d'élaborer des biomatériaux hybrides à visée ostéoarticulaire.

Etat de l'art

I. Les tissus musculo-squelettiques

Les tissus musculo-squelettiques jouent un rôle prépondérant dans la protection des organes mous et le mouvement. Ils sont affectés par des pathologies qui touchent un grand nombre de personnes et qui peuvent s'avérer extrêmement handicapantes, sans traitement réellement efficace à l'heure actuelle. Ici nous nous intéresserons aux constituants musculo-squelettiques que sont le tissu osseux, et celui-ci nécessitant une vascularisation très riche, aux vaisseaux sanguins, ainsi que le cartilage articulaire.

1. Le tissu osseux

Notre travail s'attachant à traiter les pathologies qui affectent les os, il faut en comprendre la structure et le fonctionnement, puis décrire ces pathologies.

a. Rôles et organisation

Le squelette humain comporte en moyenne 206 os dont le rôle est de protéger les organes mous internes, de fournir un support aux muscles pour permettre le mouvement, d'accueillir la moelle osseuse qui assure l'hématopoïèse, et de participer à l'homéostasie phosphocalcique. Les os sont reliés entre eux par des ligaments et des articulations, et les muscles squelettiques s'y fixent à l'aide de tendons, permettant à l'ensemble de se déplacer et de constituer l'appareil locomoteur. La structure particulière des os leur confère légèreté, souplesse et solidité, leur permettant de résister aux chocs sans alourdir outre mesure le poids du corps [1].

Les os sont classés en trois types : les os courts, comme les os du carpe, les os plats comme la scapula et enfin les os longs comme le fémur. Les os longs comportent une zone centrale appelée diaphyse constituée d'os cortical compact, creusé par le canal médullaire contenant la moelle dite jaune, riche en lipides (Figure 1). Aux extrémités des os longs se trouvent les épiphyses, qui sont constituées de tissu spongieux plus fragile, contenant la moelle dite rouge, siège de l'hématopoïèse, et entouré d'une couche d'os cortical. L'os spongieux (ou trabéculaire) est organisé en travées qui permettent de transférer les forces d'écrasement vers la diaphyse et confère une grande résistance. Entre la diaphyse et l'épiphyse se trouve la

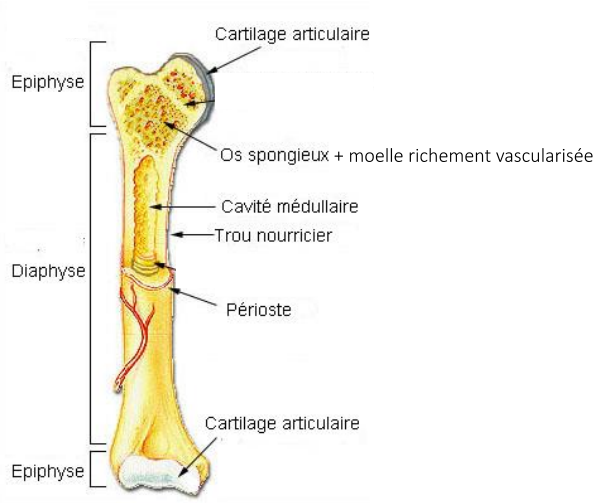

Figure 1 Représentation d'un os long. Source : https://commons.wikimedia.org/wiki/File:Os_long_1.jpg

métaphyse, zone où se trouve le cartilage de croissance chez l'enfant et qui permet l'allongement de l'os. La vascularisation de l'os est assurée par le périoste, une fine membrane riche en vaisseaux qui l'entoure.

L'os est un tissu en remodelage constant, sous le contrôle de deux types cellulaires : les ostéoblastes et les ostéoclastes. Les ostéoblastes sont d'origine mésenchymateuse et se situent à la surface de l'os en croissance. Ils synthétisent la matrice organique de l'os, constituée essentiellement de collagène de type I et favorisent la calcification de l'os. Ils synthétisent entre autres l'ostéocalcine, enzyme qui favorise l'interaction du calcium avec le phosphate formant l'hydroxyapatite, qui est le principal constituant de la matrice minérale de l'os, ou encore la phosphatase alcaline qui permet de libérer le phosphate inorganique dans le milieu extracellulaire. Les ostéoblastes, lorsqu'ils s'entourent complètement de matrice osseuse à l'intérieur d'une lacune, deviennent des ostéocytes. Ils sont alors intégrés dans le tissu osseux et communiquent avec les ostéocytes voisins grâce à des prolongements cytoplasmiques qui empruntent des canalicules dans la matrice osseuse. Les ostéoclastes quant à eux sont d'origine hématopoïétique et font partie de la lignée myéloïde. Leur rôle consiste à résorber la matrice osseuse afin d'en permettre le renouvellement : c'est le processus d'ostéolyse qui se déroule au niveau de leur zone d'ancrage. Par l'action de la pompe à proton, ils créent un milieu acide favorable à la dissolution de la matrice minérale (hydroxyapatite). Ensuite, ils libèrent des enzymes lysosomales qui vont digérer la matrice organique constituée de protéines. Les

composés libérés sont ensuite réabsorbés, participant ainsi notamment à l'homéostasie phosphocalcique et à la néosynthèse de matrice osseuse.

Le tissu osseux est également formé d'une matrice extracellulaire particulière, à la fois organique et minérale, synthétisée par les ostéoblastes. Le collagène de type I représente 90 % de la matrice organique. Il est associé à d'autres protéines non collagéniques comme l'ostéocalcine pour former le tissu ostéoïde qui sera minéralisé dans un second temps. Les fibres de collagène de type I possèdent des sites de nucléation qui permettent la formation de cristaux d'hydroxyapatite lorsque la concentration en ions calcium et phosphate est adéquate. Ainsi, le tissu osseux acquiert souplesse et solidité. Les vaisseaux sanguins qui le parcourent contribuent à la formation et à l'entretien de l'os. L'étude de la composition du tissu osseux et de sa formation doivent en effet être prises en compte dans une approche d'ingénierie tissulaire visant à le reconstituer.

b. Les vaisseaux sanguins

Le tissu osseux étant très richement vascularisé, il est nécessaire de s'intéresser également aux vaisseaux sanguins dans une approche visant à le régénérer.

Le sang, pour pouvoir assurer son rôle de tissu de soutien dans tout l'organisme, est acheminé à chaque organe et ramené aux poumons par un réseau vasculaire complexe constitué d'artères, de capillaires et de veines. La circulation sanguine est répartie en deux circuits : la circulation générale, qui achemine le sang oxygéné vers les organes, et la circulation pulmonaire, qui permet de ramener le sang appauvri en oxygène aux poumons. Chaque vaisseau est spécialisé en fonction de son rôle ou de sa localisation pour favoriser la diffusion des nutriments, de l'oxygène et des cellules dans les tissus. De plus, il doit être capable de résister à des contraintes mécaniques fortes, liées à la pression qu'exerce le sang contre la paroi notamment lorsqu'il est expulsé du cœur (la contrainte cyclique), ainsi qu'à l'écoulement du sang le long du vaisseau (les forces de cisaillement) [2]. Ainsi, l'arbre vasculaire est organisé de telle sorte qu'à la sortie du cœur, il emprunte de grosses artères élastiques qui absorbent la pression exercée par la systole, qui se subdivisent ensuite en artères musculaires puis en artérioles qui répartissent le sang dans l'organisme jusqu'au réseau de capillaires, les plus petits vaisseaux du corps qui vont assurer la diffusion de l'oxygène et des nutriments (pour la circulation générale) dans presque tous les tissus [3]. Ensuite, les veinules puis des veines de calibre de plus en plus gros vont permettre de ramener le sang jusqu'au cœur, à l'aide de valvules qui empêchent le retour du sang.

Ainsi, la paroi de chaque vaisseau est constituée de telle sorte qu'elle lui confère ses propriétés en fonction de sa localisation dans l'arbre vasculaire. Cette paroi a une organisation commune à presque tous les vaisseaux, à l'exception du réseau capillaire constitué de micro-vaisseaux (Figure 2). Elle est organisée en tuniques concentriques qui sont, de la plus interne à la plus externe : l'intima, la media et l'adventice [4]. L'intima et la media sont séparées par la limitante élastique interne, tandis que la media et l'adventice sont séparées par la limitante élastique externe. Ces limitantes sont toutes deux de fines couches de fibres élastiques acellulaires.

L'intima est la couche la plus interne et la plus fine : elle est constituée d'une monocouche de cellules endothéliales (CE) fixées sur une lame basale. Elle est directement au contact du sang, et les CEs qui la constituent ont donc pour rôle d'assurer l'étanchéité du vaisseau, tout en laissant traverser certaines cellules (notamment immunitaires) ainsi que les nutriments et l'oxygène. De plus, les CEs supportent la contrainte de cisaillement et synthétisent du monoxyde d'azote (NO) en réponse à celle-ci. Le NO joue un rôle dans la vasodilatation du vaisseau en réponse au cisaillement. Les CEs inhibent également l'agrégation plaquettaire et préviennent ainsi la formation de caillots dans le vaisseau sain.

La media est la tunique intermédiaire du vaisseau. Elle est constituée de couches concentriques de cellules musculaires lisses (CML) associées à des fibres extracellulaires telles que le collagène, l'élastine ou des protéoglycanes. Les CML assurent la contraction et la dilatation du vaisseau en réponse aux signaux vasoconstricteurs ou vasodilatateurs. D'une part, ces signaux sont issus du système nerveux autonome : sympathique (vasoconstricteurs) ou parasympathique (vasodilatateur) dont les fibres nerveuses. D'autre part, les CML répondent également à certaines hormones : c'est le cas notamment du NO produit par les CE ou d'autres hormones comme l'adrénaline, la vasopressine ou l'angiotensine. La composition relative en fibres extracellulaires dépend de la structure et de la fonction du vaisseau : par exemple, les artères élastiques telles que l'aorte, qui sont proches du cœur, sont riches en fibres élastiques afin de résister à la pression engendrée par la contraction du ventricule.

L'adventice est la tunique externe du vaisseau : elle est constituée essentiellement de fibroblastes et de fibres de collagène. L'adventice permet l'ancrage du vaisseau dans les tissus environnants en plus d'assurer le rôle de nutrition du vaisseau. Dans ceux de plus gros diamètre elle est traversée par les vaisseaux (*vasa vasorum*) et les nerfs (*nervi vasorum*) permettant respectivement d'irriguer et d'innerver le vaisseau.

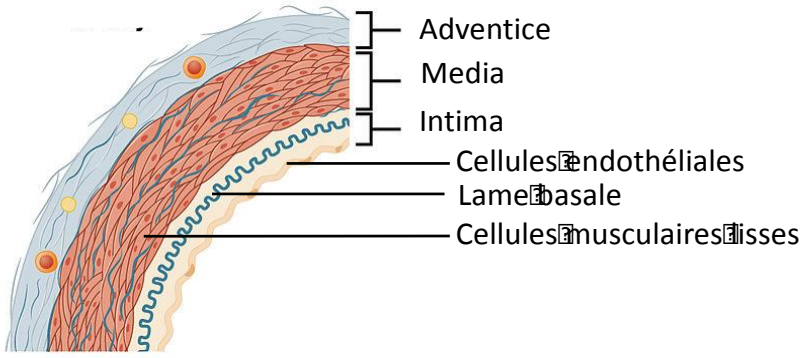

Figure 2 : Représentation schématique d'une artère avec ses trois tuniques.
Source : https://commons.wikimedia.org/wiki/File:2103_Muscular_and_Elastic_Artery_Arteriole.jpg
Auteur : OpenStax College

Dans les os longs, la vascularisation est assurée dans la partie interne majoritairement par une artère nourricière qui pénètre dans la diaphyse par le foramen nourricier. Elle se divise rapidement en branches ascendantes et descendantes qui se dirigent vers chacune des épiphyses de l'os, et qui finissent par former en se subdivisant à nouveau un réseau capillaire extrêmement dense dans la moelle osseuse permettant, en plus de l'apport en oxygène et en nutriment, le remodelage osseux en permettant aux précurseurs ostéoblastiques et ostéoclastiques de se déplacer vers les sites de remodelage. Des artères épiphysaires participent également à l'irrigation des épiphyses. Le sang quitte ensuite le réseau capillaire médullaire par un sinus veineux central qui draine les capillaires sur toute la hauteur de la cavité médullaire et qui aboutissent dans la veine nourricière qui quitte l'os par le foramen nourricier. La vascularisation des parties les plus externes est assurée par les vaisseaux parcourant le périoste. Ainsi notre travail s'articule entre ces deux acteurs que sont la vascularisation et la formation ostéochondrale.

c. Pathologies osseuses

Les pathologies affectant les os ont de nombreuses origines : congénitale, traumatique, métabolique, infectieuse, ou néoplasique. Une des pathologies osseuses les plus représentées est l'ostéoporose : elle touche 30 % des femmes et 10% des hommes, et affecte plus particulièrement les personnes de plus de 60 ans. Cette pathologie est liée à une diminution de

l'épaisseur des travées et de l'os cortical, ainsi qu'à une modification de l'architecture des travées de l'os spongieux. Cette maladie touche plus particulièrement les femmes en raison de la carence œstrogénique qui suit la ménopause, entraînant une augmentation de la résorption osseuse. Elle augmente significativement le risque de fractures, particulièrement au niveau des vertèbres ou des os longs (col du fémur par exemple).

Lorsque le tissu osseux est fracturé, ce qui résulte généralement d'un traumatisme, il est capable de se régénérer spontanément dans des conditions favorables, grâce à l'action de remodelage constant des ostéoblastes et ostéoclastes [5]. Cependant, lorsque la fracture dépasse une taille critique, l'os n'est plus capable de se régénérer convenablement. Dans ce cas, le chirurgien doit alors réaliser une greffe de tissu osseux, qui peut être autologue ou allogénique. L'autogreffe est idéale car elle permet d'insérer dans le site lésé un tissu qui provient du patient lui-même, avec d'excellentes propriétés ostéoconductives (la capacité de l'os à se former sur une surface), ostéoinductives (la capacité du tissu à recruter des progéniteurs des cellules osseuses) et ostéogéniques (la capacité à induire la différenciation des progéniteurs en ostéoblastes). Cependant, elle entraîne des dommages secondaires au niveau du site donneur, et le tissu peut ne pas être présent en quantité suffisante. La greffe allogénique permet d'utiliser des tissus présents dans une banque, mais qui ont en général perdu leurs propriétés ostéoinductives [6]. Les inconvénients de l'allogreffe sont que le tissu du donneur peut ne pas être suffisant pour combler la fracture, en plus des problèmes liés au risque de transmission de maladies ou de rejet de la greffe.

Il existe donc un besoin en tissu osseux fabriqué par le biais de l'ingénierie tissulaire, en quantité suffisante pour combler des fractures de grande taille, mais répondant également à un cahier des charges exigeant : le greffon doit posséder des propriétés ostéoconductives, ostéoinductives et ostéogéniques et être richement vascularisé [7], en plus de ne pas poser de problèmes de compatibilité en vue de la greffe. Notre travail doit donc tenir compte de chacun de ces paramètres.

2. L'articulation

Le travail présenté ici s'attache également à répondre à certaines pathologies qui atteignent le cartilage articulaire, il nous faut donc décrire également l'articulation pour mieux comprendre comment la réparer.

a. Rôle et organisation

Les articulations qui nous intéressent dans ce travail sont les articulations synoviales, comme celle du genou. Elles réunissent à l'aide de ligaments deux pièces osseuses recouvertes de cartilage hyalin qui empêche l'usure de l'os, et sont entourées d'une capsule articulaire remplie de liquide synovial qui joue le rôle de lubrifiant (Figure 3). Le cartilage existe sous plusieurs formes : le cartilage hyalin et non hyalin. Le cartilage hyalin se retrouve particulièrement au niveau articulaire où il a pour rôle de supporter le poids de l'organisme et de faciliter le mouvement des os les uns par rapport aux autres. Le cartilage articulaire est en contact et forme une continuité avec l'os sous-chondral qui est vascularisé. Parmi les cartilages non hyalins, on retrouve le cartilage élastique situé notamment au niveau du pavillon de l'oreille ou de l'épiglotte, et le fibrocartilage qui constitue en partie la trachée et la symphyse pubienne. Le cartilage hyalin articulaire subit de fortes contraintes de compression (liées au poids du corps) et de cisaillement (provoquées par le mouvement de l'articulation). Il est avasculaire et non innervé et se nourrit donc seulement par diffusion à partir de l'os sous-chondral. Les cellules présentes dans le cartilage sont les chondrocytes. Ils rassemblés en groupes isogéniques (issus d'un même chondroblaste) dans des lacunes, formant des unités appelées chondrones. La matrice extracellulaire du cartilage est essentiellement composée de fibres de collagène de type II et de protéoglycanes associés à de l'acide hyaluronique. Cette composition confère au cartilage une grande élasticité et une grande résistance à la compression indispensable au

fonctionnement de l'articulation. Notre travail doit donc également prendre en compte la composition et la structure du cartilage articulaire.

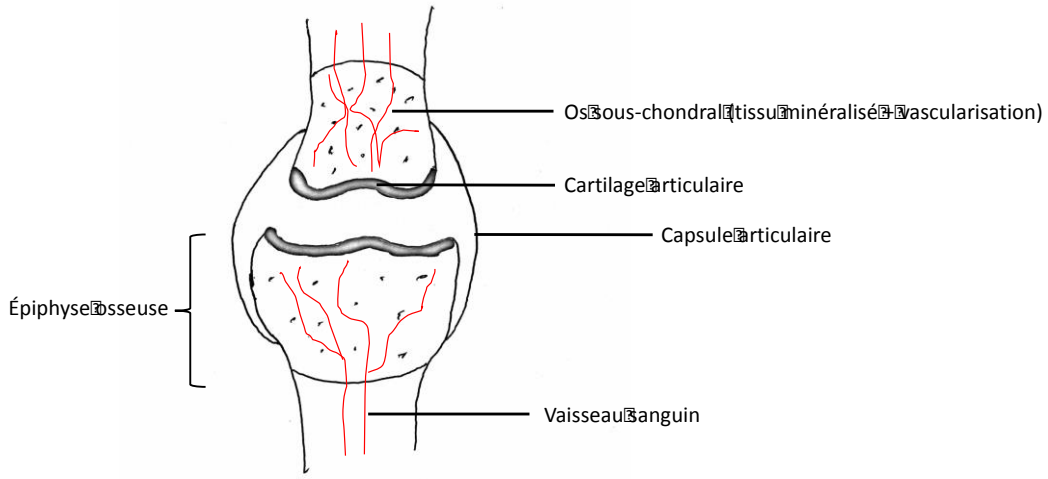

Figure 3 : Représentation schématique d'une articulation synoviale

b. Pathologies articulaires

Les principales pathologies affectant l'articulation sont liées au cartilage et sont le traumatisme et les maladies dégénératives comme l'arthrose. Dans un cas comme dans l'autre, si le tissu cartilagineux s'amincit et ne peux plus accomplir son rôle, les pièces osseuses de l'articulation finissent par frotter l'une contre l'autre et se détériorer. Ceci est la cause de douleurs et de perte de mobilité de l'articulation, pouvant aboutir à une arthrite inflammatoire dégénérative [8]. Lorsque la dégénération du cartilage est trop avancée, la seule voie possible est le remplacement total de l'articulation par une prothèse [9]. Ici encore, l'ingénierie tissulaire tente donc de reconstruire le tissu cartilagineux, car par sa nature avasculaire il est incapable de se régénérer seul. Notre travail vise donc à prendre en compte tous ces facteurs pour traiter les lésions atteignant le cartilage articulaire.

II. L'ingénierie tissulaire

Le travail présenté ici repose sur une approche d'ingéniérie tissulaire, il faut donc en aborder ici les concepts, ainsi que les acteurs en jeu dans ce domaine.

1. Définition

À ce jour, les pathologies affectant les articulations ou les os n'ont pas de traitements totalement efficaces, c'est à dire de solutions qui pourraient permettre d'apporter une restauration complète des fonctions perdues. La médecine régénératrice est une discipline qui vise à restaurer des organes ou des tissus lésés par une maladie ou un traumatisme. Elle s'appuie sur deux concepts complémentaires : la thérapie cellulaire et l'ingénierie tissulaire. La thérapie cellulaire vise à utiliser des cellules pour pallier la déficience du tissu et le régénérer. Dans ce cas, des cellules souches ou matures peuvent être employées. Les cellules souches ont la propriété de pouvoir se diviser en grand nombre et de se différencier vers des phénotypes cellulaires spécialisés en fonction de leur origine, ce qui leur confère un grand intérêt pour l'utilisation en thérapie cellulaire.

L'ingénierie tissulaire cherche à régénérer un tissu ou à en reproduire le fonctionnement en associant trois éléments : des cellules souches ou matures, un biomatériau qui leur sert de support (mais qui peut également avoir d'autres propriétés intrinsèques), et des stimuli chimiques, biologiques et/ou mécaniques. Ces trois éléments forment la triade de l'ingénierie tissulaire et doivent être sélectionnés avec soin pour conférer à l'ensemble une unité

fonctionnelle répondant aux besoins du tissu à reconstruire. Nous allons maintenant aborder chacun de ces facteurs car ils sont pris en compte dans notre travail.

a. Les cellules

Les cellules étant généralement l'unité fonctionnelle de base d'un tissu, elles sont généralement placées au centre d'une approche d'ingénierie tissulaire. Elles sont ensemencées sur un biomatériau adapté qui leur sert de support. Les cellules utilisées dans ce cas peuvent provenir du patient lui-même (cellules autologues, [10]), d'un donneur (cellules allogéniques, [11]), ou plus rarement d'une autre espèce (cellules xénogéniques, [12]). L'utilisation de cellules matures provenant du patient pose problème car elles proviennent en général d'un tissu déjà affecté par une maladie, ou leur prélèvement est susceptible de l'aggraver. Le prélèvement chez un individu sain présente lui aussi le risque de déclencher une pathologie, liée soit au manque engendré par le prélèvement de cellules, soit à une réaction déclenchée par la procédure du prélèvement qui est généralement chirurgicale. De plus, la plupart des cellules matures spécialisées ont perdu leur capacité de division, et perdent rapidement leur phénotype *in vitro* [13][14]. L'utilisation de cellules souches capables de se diviser et de se différencier est donc rapidement devenue centrale en médecine régénératrice. Les cellules souches mésenchymateuses (CSM) présentent dans ce contexte un très grand intérêt. Elles sont capables de se diviser en grand nombre *in vitro* et de fournir une grande quantité de cellules fonctionelles. Elles peuvent se différencier vers tous les types cellulaires mésenchymateux comme les adipocytes, les chondrocytes ou les ostéocytes [15]. Elles sont retrouvées et peuvent être isolées à partir d'un grand nombre de tissus comme les follicules capillaires [16], le cordon ombilical, le tissu adipeux , la moelle osseuse [17], ou la pulpe dentaire [18]. De plus, les CSM ont des propriétés immunomodulatrices qui revêtent un grand intérêt dans un contexte de greffe allogénique [19]. Il a également été démontré que leurs capacités régénératrices en thérapie cellulaire passent par leurs produits de sécrétion dont les vésicules extracellulaires (*cf*. article en Annexe). Les CSM de la gelée de Wharton (CSM-GW), le tissu conjonctif entourant les vaisseaux du cordon ombilical, présentent l'avantage de pouvoir être obtenues de manière non invasive. Elles sont disponibles en grand nombre, et sont plus immatures d'un point de vue immunologiques que leurs semblables issues de tissus adultes[20]. Aussi, le groupe d'ingénierie vasculaire du laboratoire IMoPA a déjà démontré leur capacité à se différencier vers des phénotypes vasculaires comme les cellules endothéliales [19][21]. L'utilisation de CSM-GW différenciées a donc pour **avantage** de s'affranchir des contraintes liées aux

prélèvements autologues (invasivité, vieillissement cellulaire, perte de phénotype des cellules matures…). Cependant, **l'inconvenient** est que des cellules allogéniques sont tout de même employées, et que la différenciation cellulaire peut être difficile à obtenir car elle demande la maîtrise de nombreux facteurs.

b. Les biomatériaux

Les cellules dans un tissu s'entourent d'une matrice extracellulaire (MEC) qui non seulement leur sert de support, mais qui confère également au tissu ses propriétés chimiques, physiques et biologiques. Dans une approche d'ingénierie vasculaire, les biomatériaux ont pour but de se rapprocher de la structure de la MEC native du tissu à réparer, afin de guider la différenciation cellulaire et de supporter les contraintes physiologiques liées à leur implantation chez le malade. Il permet à la culture cellulaire d'avoir lieu en trois dimensions, et d'éviter les problèmes de dédifférenciation fréquemment rencontrés dans les cultures en monocouche *in vitro*. Ses propriétés mécaniques jouent également un rôle important, car il est susceptible de devoir supporter des contraintes mécaniques intenses : par exemple la pression du sang dans un vaisseau, la compression dans le cartilage ou l'écrasement dans l'os.

Les biomatériaux peuvent être d'origine synthétique [22] ou naturelle. Parmi les biomatériaux synthétiques, sont déjà couramment utilisés en santé des métaux comme le titane pour les prothèses de hanche, les céramiques pour les prothèses dentaires, ou les polymères pour les lentilles de contact ou les colles chirurgicales. **L'inconvénient** de ce type de matériau est qu'il interagit peu l'environnement du site d'implantation. Les biomatériaux d'origine naturelle peuvent être issus de polysaccharides comme le chitosan (dérivé de la chitine présente dans la carapace des crustacés)[23], l'alginate (issu des algues brunes)[24] ou la cellulose végétale [25], qui ont des structures très proches des composants de la MEC humaine. Dans les biomatériaux d'origine naturelle sont également retrouvés les MEC décellularisées, qui proviennent de toutes sortes de tissus : la rate [26], la cornée [27], le cartilage [28] ou l'os [29] en sont autant d'exemples. **L'avantage** d'employer ce type de matrices est qu'il reproduit au plus près la MEC du tissu à régénérer puisqu'elles sont synthétisées *in vivo* par des cellules humaines. L'inconvénient réside dans la nécessité de prélever ses matrices, souvent de manière invasive. Le groupe ingénierie vasculaire de l'UMR IMoPA a démontré la possibilité d'utiliser la MEC issue de la gelée de Wharton (MEC-GW) comme biomatériau utilisable pour l'ingénierie tissulaire (*cf*. article suivant).

Publication n°1 : Article scientifique

Résumé en français

Matrice dérivée du cordon ombilical humain : un biomatériau adapté pour des applications en ingénierie tissulaire.

Pan Dan, Émilie Velot, Benjamin Mesure, Guillaume Groshenry, Jalal Bacharouche, Véronique Decot et Patrick Menu

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Un des axes de recherche en médecine régénératrice consiste à développer des matrices extracellulaires (MEC) capables de mimer l'environnement naturel des cellules. Dans ce but, les biomatériaux synthétiques comme naturels ont été largement étudiés. Les polymères naturels présentent un plus grand intérêt car ils présentent moins de problèmes de biocompatibilité. Le collagène et la fibronectine sont bien connus et utilisés comme support de culture cellulaire dans de nombreux modèles d'ingénierie tissulaire. En tant que protéine la plus abondante protéine chez les mammifères, le collagène peut être obtenu à partir de tissus animaux ou humains. Actuellement, le collagène commercial est classiquement extrait de la queue de rat ou du placenta humain. En raison des problèmes de rejet xénogéniques, la MEC animale a peu d'application cliniques et cela favorise le développement de produits dérivés de tissus humains. Le placenta humain contient une matrice contenant de nombreux facteurs de croissance ayant un rôle dans l'angiogenèse. Le lysat plaquettaire est une autre source prometteuse d'additif de culture de grade clinique pour l'expansion cellulaire. Ces découvertes encouragent la recherche de nouvelles sources de tissus humains pour l'ingénierie tissulaire.

Cette étude visait à explorer la matrice extracellulaire issue du tissu conjonctif du cordon ombilical aussi appelé gelée de Wharton (MEC-GW), connue pour héberger une grande concentration de cytokines. Nous avons utilisé une méthode enzymatique pour isoler et solubiliser la MEC-GW, caractérisé le produit obtenu et examiné son potentiel en ingénierie tissulaire.

La MEC-GW a été obtenue à partir de cordons ombilicaux découpés en petits morceaux et digérés dans de la trypsine pendant 24h jusqu'à obtention d'une solution visqueuse. La concentration en MEC-GW dans cette solution a été déduite en lyophilisant une partie de la solution. Un kit d'analyse des facteurs de croissance de l'angiogenèse a révélé une forte concentration en facteurs de croissance et protéines matricielles tels que le vascular endothelial

growth factor (VEGF), le fibroblast growth factor (FGF-2), le platelet derived growth factor-AA (PDGF-AA), le transforming growth factor- beta 1 (TGF-ß1) et du collagène de type XVIII. La présence de collagène de type I a également été mise en évidence dans la MEC-GW par Western Blot, collagène qui est hydrolysé par la trypsine en absence de neutralisation par du sérum de veau fœtal (SVF). De plus, aucune présence d'ADN résiduel n'est détectée au terme de la préparation de la MEC-GW, indiquant que la décellularisation est complète. Il a également été démontré que la MEC-GW peut se déposer et former une couche de 45 nm d'épaisseur visible en microscopie de force atomique sur des lames de verre, ainsi que sur des artères de cordon ombilical désendothélialisées. Ce dépôt de MEC-GW est capable de supporter l'adhésion et la prolifération de CSM-GW et de cellules endothéliales de la veine du cordon ombilical humain (HUVEC).

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Human umbilical cord derived matrix: A scaffold suitable for tissue engineering application

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Abstract.
BACKGROUND: Human tissue derived natural extracellular matrix (ECM) has great potential in tissue engineering.
OBJECTIVE: We sought to isolate extracellular matrix derived from human umbilical cord and test its potential in tissue engineering.
METHODS: An enzymatic method was applied to isolate and solubilized complete human umbilical cord derived matrix (hUCM). The obtained solution was analyzed for growth factors, collagen and residual DNA contents, then used to coat 2D and 3D surfaces for cell culture application.
RESULTS: The hUCM was successfully isolated with trypsin digestion to acquire a solution containing various growth factors and collagen but no residual DNA. This hUCM solution can form a coating on 2D and 3D substrates suitable cell culture.
CONCLUSION: We developed a new matrix derived from human source that can be further used in tissue engineering.

Keywords: Human matrix, tissue engineering, growth factors, residual DNA, stem cells

1. Introduction

One of the focus to generate new biomaterials in regenerative medicine is the development of multifunctional extracellular matrix (ECM) that can mimic a natural environment for cells [1]. To do so, both synthetic and natural polymers have been widely investigated [2]. Natural polymers draw much attention because they usually have less concern about biocompatibility. Collagen and fibronectin are well known and applied in various tissue engineering applications to support cell culture. As the most abundant protein in mammals, collagen can be derived from animal or human tissue. Currently, commercial collagen is usually from rat-tail or human placenta [3,4]. With the concern of xenogeneic rejection, animal de-

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rived ECM has no clinical potential and this has led to develop human derived product. Human placenta can generate matrix containing numerous growth factors that have function in angiogenesis [5]. Platelet lysate is another promising source that can be used as clinical-grade supplement for cell expansion [6]. These achievements encourage the sighting of new human tissue sources for tissue engineering.

This study aims to explore human umbilical cord derived matrix (hUCM), which was known to contain huge amount of cytokines [7]. We used an enzymatic method to isolate and solubilized hUCM, characterized the obtained product and assessed its potential in tissue engineering.

2. Methods

2.1. Human umbilical cord derived matrix preparation

Human umbilical cords were collected at the delivery suite at regional maternity hospital of Nancy at the University of Lorraine in accordance with TCG/11/R/011 within 12 h of birth. The whole umbilical cord was cut into small pieces and the connective tissue was dissociated. Umbilical cord connective tissue (2–3 g, wet weight) was digested in 10 mL of trypsin (0.025%) at 37°C for 24 h with magnetic stirring. The stirred suspensions were centrifuged at 16,000g for 10 min. The collected supernatants were filtered (100 μm) and 5 mL of fetal bovine serum (FBS) was added to inhibit trypsin. PBS was added to have a 20 mL final solution. The obtained hUCM solution was conserved at 4°C for further use. The precise concentration of hUCM was calculated as follows: 10 mL of the prepared hUCM solution were lyophilized; 10 mL of solution containing 5 mL of trypsin, 2.5 mL of FBS and 2.5 mL of PBS were also lyophilized. The concentration of hUCM (mg/mL) was calculated by the dry powder weight of total hUCM minus the weight of lyophilized trypsin–FBS–PBS solution.

2.2. Characterization of the hUCM solution

The hUCM solution generated by trypsin digestion was characterized for its cytokine contents by an angiogenesis array kit (R&D systems, France) according to the manufacture's protocol. Detected spots were analyzed with Image J software for semi-quantitative measurement. Western-blot was performed to detect collagen I in the solution. Rat-tail collagen I was used as a positive control and the trypsin–FBS–PBS mixture as negative control.

In order to check whether the hUCM solution contains residual DNA after trypsin treatment, agarose gel electrophoresis (10%) was performed. The trypsin–FBS–PBS mixture was used as a control. We also assessed the influence of FBS on DNA stability by preparing hUCM solution without adding FBS.

2.3. Elaboration and characterization of the hUCM coating for cell culture

hUCM solution was used to form a coating for cell culture. For 2D coating, the solution was deposited on glass coverslips that were previously treated with SDS–HCl. The glass coverslips were incubated at 37°C for 1 h before being washed twice with PBS. For 3D coating, hUCM solution was injected into de-endothelialized umbilical artery and performed similarly to 2D coating. Atomic force microscopy was employed to evaluate the coating.

2D coating was used to assess its potential for cell culture. Human mesenchymal stem cells and human umbilical vein endothelial cells were isolated according to El Omar et al. [8] and cultured on hUCM

coating for 5 days before being marked with phalloidine and DAPI for fluorescent microscopy observation as previously described [9].

3. Results

3.1. Characterization of human umbilical cord matrix

After 24 hours of magnetic stirring in presence of trypsin, hUCM was digested into a viscous solution (Fig. 1). The concentration of hUCM was then precisely calculated using a lyophilized solution. The influence of trypsin, FBS and PBS was also taken into account during calculation. A cytokine array focused on soluble factors revealed that the solution contained abundant growth factors and ECM proteins, including vascular endothelial growth factors (VEGF), fibroblast growth factors-2 (FGF-2), platelet derived growth factor-AA (PDGF-AA) and transforming growth factor-beta 1 and collagen XVIII (Fig. 2(a)). Collagen I was also detected by western blot in three independently prepared hUCM. We also observed that the neutralization of trypsin by FBS is mandatory, since collagen I was destroyed in absence of trypsin neutralization (Fig. 2(b)).

3.2. Residual DNA detection

The presence of residual human DNA was next determined. DNA was detected in samples without FBS, but residual DNA was destroyed when FBS was added. Thus, in all the three independently prepared full hUCM, no residual DNA was detected (Fig. 2(c)).

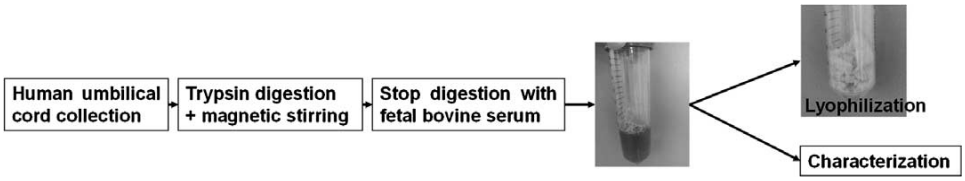

Fig. 1. Human umbilical cord matrix was obtained by trypsin digestion under magnetic stirring, followed by adding FBS. The solution was then lyophilized for precise concentration calculation or further biological characterization.

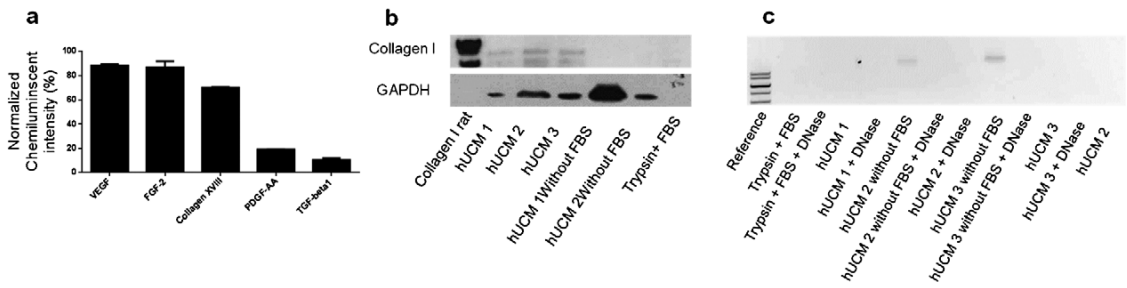

Fig. 2. Characterization of hUCM solution. (a) Biochemical analysis using human angiogenesis array demonstrated growth factors and collagen content. (b) Western-blot using collagen I antibody indicated that hUCM contained collagen I. (c) Residual DNA was determined by agarose gel electrophoresis. No residual DNA was detected in any of the three independently prepared hUCM.

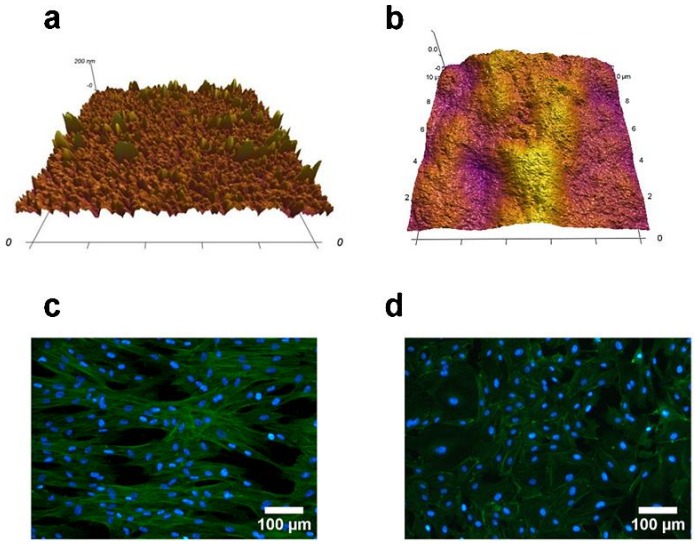

Fig. 3. A coating can be formed by hUCM on 2D substrate (a) and 3D substrate. The 2D coating can support cell adhesion of human mesenchymal stem cells (c) and human umbilical vein endothelial cells (d). (Colors are visible in the online version of the article.)

3.3. Cell culture coating derived from hUCM

On 2D glass coverslips, hUCM can form a coating which had a uniform distribution with thickness of 45 ± 12 nm. As assessed by AFM, hUCM can also coat 3D de-endothelialized umbilical artery (Fig. 3(a) and (b)).

Human mesenchymal stem cells and endothelial cells seeded on coated glass slides were showed to adhere as observed on fluorescent images indicating that the new coating is able to support cell adhesion and proliferation (Fig. 3(c) and (d)).

4. Discussion

The discovery of new human derived source for fabricating biomaterials remains a goal to reach in tissue engineering [10]. Besides placenta which is considered as an important and useful source for human derived extracellular matrix for tissue engineering, other sources of healthy human tissues are currently explored. This study reports an enzymatic method to isolate a complex matrix from human umbilical cord. This matrix derived from umbilical cord contained abundant growth factors, and extracellular protein such as collagen. These components clearly provide a suitable environment for stem cells and mature cells adherence and growth. Umbilical cord is considered as a niche for mesenchymal stem cells [11]. When used as a matrix source, the isolation procedure will destroy cells in the tissue and lead to the release of cell DNA. The residual human DNA may induce a host immune response. Only biomaterials which do not contain residual DNA may have a clinical transfer potential [12]. In our study, the procedure was ended by adding FBS to inhibit trypsin reaction. Interestingly, FBS was reported to contain heat-stable nucleases that can eliminate residual DNA [13]. This advantage of FBS allows us to obtain a hUCM without any residual DNA, which indicate the hUCM may be safely used in human

regenerative medicine. However, we cannot exclude the risk of xenogeneic reaction due to presence of FBS, even if the percentage used was low. An alternative of FBS will be required to supply a full clinical grade hUCM.

5. Conclusion

Our results revealed an enzymatic method to obtain a complex matrix from human umbilical cord. The hUCM contained various growth factors and different types of collagen, which can provide a biomimetic environment for cell culture. No residual DNA was detected from the hUCM, which makes it a safe product for clinical transfer. The hUCM can easily form a coating on 2D and 3D substrates to support cell culture. This point makes hUCM a strong tool for tissue engineering.

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Conflict of interest

The authors have no conflict of interest to report.

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c. Les stimuli

Les cellules perçoivent directement les conditions biologiques, chimiques ou physiques du milieu qui les entourent, et ces les caractéristiques dépendent du tissu étudié. Ces facteurs extrinsèques influencent le comportement des cellules et agissent comment de véritables stimuli de l'environnement qui les poussent à s'adapter et se différencier pour y répondre. C'est pourquoi une approche d'ingénierie tissulaire doit nécessairement les prendre en compte et les intégrer dans son modèle.

Les facteurs de croissance, les cytokines et les hormones en général participent à la communication cellulaire et agissent comme des signaux qui poussent les cellules à se diviser, migrer, se différencier et/ou changer leur métabolisme. Ainsi, de nombreuses études utilisent déjà des facteurs biochimiques pour guider le comportement des cellules dans la direction souhaitée [30]. D'autre part, les cellules sont également sensibles aux contraintes mécaniques de leur environnement, c'est par exemple le cas des cellules vasculaires qui subissent des contraintes d'étirement, ou des chondrocytes qui reçoivent des forces de compression intenses. Ces paramètres mécaniques sont aujourd'hui également intégrés dans de nombreuses études d'ingénierie cellulaire [2][31]. La compostion de la MEC entourant les cellules doit également être prise en compte dès les étapes *in vitro* afin de permettre de se diriger vers un implant en 3 dimensions transférable *in vivo*.

d. La thérapie génique dans la réparation tissulaire

L'utilisation de molécules recombinantes en culture présente des **inconvénients** liés notamment à la demie-vie courte de ces molécules dans le milieu. Depuis plusieurs années, la thérapie génique se présente comme la solution à ce problème en employant des gènes thérapeutiques. Elle s'appuie sur l'utilisation de vecteurs afin d'introduire ces gènes thérapeutiques dans des cellules, qui sécrètent alors elles-mêmes les facteurs de différenciation par exemple. Les vecteurs utilisés en thérapie génique peuvent être viraux ou non-viraux. Les vecteurs non viraux les plus utilisés sont les plasmides, introduits à l'intérieur des cellules par l'intermédiaire d'agents chimiques ou physiques permettent de rendre les membranes perméables. **L'avantage** de ce type de vecteur est qu'il n'est pas introduit dans le génome de l'hôte, présentant ainsi peu de risque liés à l'intégration. En revanche, **l'inconvénient** est qu'il présente une efficacité faible, en effet avec les plasmides le nombre de cellules transfectées reste faible. Les vecteurs viraux tels que les lentivirus ou les adénovirus sont en revanche plus

efficaces, permettant d'obtenir un niveau de transduction plus élevé et surtout une plus grande persistance de l'expression dans le temps, mais il présente un risque intégratif lié à l'insertion de gènes dans l'ADN de la cellule hôte. Les vecteurs viraux et non viraux ont démontré leur efficacité dans la réparation tissulaire du cartilage [32][33] et de l'os [34][35], présentant donc ainsi un intérêt dans notre travail en permettant l'expression à long terme de facteurs de différenciation du cartilage ou de l'os, comme par exemple Sox9, un facteur de transcription central dans la différenciation chondrocytaire et la formation du cartilage [36].

2. L'ingénierie tissulaire musculo-squelettique.

L'ingénierie tissulaire a pour but de régénérer les tissus. Dans notre travail, elle s'intéresse aux tissus musculosquelettiques dont font partie l'os et le cartilage, affectés par de nombreuses pathologies touchant de plus en plus de personnes, notamment parce qu'elles sont liées au vieillissement de la population, et encore à l'absence de traitement totalement efficace. Les techniques mises en place pour recréer ces tissus font appel aux nombreux progrès effectués ces dernière décennies dans les domaines de l'ingénierie tissulaire sur l'utilisation de CSM et de biomatériaux innovants, mais également de la thérapie génique, et associent ces concepts dans des études de plus en plus complexes. Aussi, nous avons rédigé une mini-revue synthétisant les études de ces dernières années associant l'utilisation de CSM, de thérapie génique et de biomatériaux dans le but de régénérer les tissus osseux et cartilagineux, puisque c'est précisément ce type de modèle qui est à l'étude dans notre travail.

Publication n°2 : Mini-revue

Résumé en français

Biomatériaux et Thérapie génique : une combinaison pertinente pour l'ingénierie musculosquelettique employant des CSM.

Benjamin Mesure, Patrick Menu, Jagadeesh Kumar Venkatesan, Magali Cucchiarini et Émilie Velot.

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Les pathologies musculosquelettiques, particulièrement celles qui affectent les os et les articulations, demeurent un défi pour la médecine régénératrice. Les principales difficultés liées à l'ingénierie tissulaire osseuse sont la taille des lésions, le besoin d'une vascularisation riche et la synthèse des composants de la MEC appropriés dans le tissu cible. Le cartilage est lui un tissu avasculaire et possède donc des capacités de régénération très limitées. Grâce à leur capacité d'auto-renouvellement, leur plasticité et leurs propriétés immunomodulatrices, les cellules souches mésenchymateuses (CSM) sont devenues un acteur central dans l'ingénierie tissulaire, et il a déjà été démontré qu'elle étaient capables de se différencier vers des phénotypes chondrogéniques ou ostéogéniques. Qu'ils soient d'origine synthétique (par exemple le phosphate tricalcique) ou d'origine naturelle (par exemple l'acide hyaluronique), les biomatériaux peuvent être conçus afin de s'intégrer dans des lésions osseuses ou cartilagineuses pour en assurer la stabilité mécanique, mais aussi pour contrôler la distribution spatiale des cellules ainsi que leur différenciation. Des facteurs de croissance solubles sont généralement utilisés pour favoriser la différenciation cellulaire et pour stimuler la production de MEC, dans le but d'obtenir le tissu souhaité. Cependant, à cause de leur demi-vie courte, des transfections utilisant des ADN plasmidiques ou des transductions via un vecteur viral de gènes thérapeutiques peuvent être utilisées pour induire la sécrétion cellulaire de ces facteurs, permettant d'obtenir des effets plus pérènnes. De plus, le phénotype chondrocytaire peut s'avérer difficile à contrôler dans le temps, avec par exemple une dérive vers la production de marqueurs hypertrophiques ou ostéogéniques indésirables pour la production de cartilage hyalin. C'est pourquoi les stratégies de régénération de tissus sont de plus en plus élaborées, avec des associations incluant les bienfaits des CSM, des biomatériaux, et de la thérapie génique afin d'obtenir une régénération tissulaire optimale. Cette minirevue s'intéresse donc aux études

in vitro, *in situ* et *in vivo* combinant les biomatériaux et la thérapie génique associées aux CSM pour l'ingénierie cartilagineuse et osseuse.

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Biomaterials and gene therapy: a smart combination for MSC musculoskeletal engineering

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Abstract: Musculoskeletal pathologies, especially those affecting bones and joints, remain a challenge for regenerative medicine. The main difficulties affecting bone tissue engineering are the size of the defects, the need for blood vessels and the synthesis of appropriate matrix elements in the engineered tissue. Indeed, the cartilage is an avascular tissue and consequently has limited regenerative abilities. Thanks to their self-renewal, plasticity and immunomodulatory properties, mesenchymal stem cells (MSCs) became a central player in tissue engineering, and have already been shown to be able to differentiate towards chondrogenic or osteogenic phenotypes. Whether synthetic (*e.g.* tricalcium phosphate) or from natural sources (*e.g.* hyaluronic acid), biomaterials can be shaped to fit into bone and cartilage defects to ensure mechanical resistance and may also be designed to control cell spatial distribution or differentiation. Soluble factors are classically used to promote cell differentiation and to stimulate extracellular matrix synthesis to achieve the desired tissue production. But as they have a limited lifetime, transfection using plasmid DNA or transduction via viral vector of therapeutic genes to induce the cell secretion of these factors allows to have more lasting effects. Also, the chondrocyte phenotype may be difficult to control over time, with for example the production of hypertrophic or osteogenic markers that is undesirable in hyaline cartilage. Thus, tissue regeneration strategies became more elaborated, with an attempt at associating the benefits of MSCs, biomaterials, and gene therapy to achieve a proper tissue repair. This minireview focuses on *in vitro* and *in vivo* studies combining biomaterials and gene therapy associated with MSCs for bone and cartilage engineering.

Keywords: musculoskeletal lesions, MSCs, gene therapy, biomaterials, bone tissue engineering, cartilage tissue engineering.

1. INTRODUCTION

A variety of human pathologies of the musculoskeletal system remain of strong concern, showing the necessity for novel therapeutic strategies that might be based on tissue engineering approaches. Each tissue has its own characteristics that need to be taken into account when trying to build an engineered graft.

Bone pathologies following trauma, cancer, or congenital diseases require large volumes of tissue because of the size of the defects [1]. In such cases, autologous grafts are standard treatments but with intrinsic limitations often because the patient has an altered donor site. Allografts and synthetic materials also cannot offer satisfying results because of the lack of donor tissues, biocompatibility issues, or erosion. The vascularization of the bone is also a challenge as numerous vessels are required to bring sufficient oxygen and nutrients as well as progenitor cells [2,3].

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In the case of the articular cartilage, the most represented causes of degradation are trauma and degenerative diseases such as osteoarthritis (OA). Because of its avascular and aneural nature, the cartilage has a poor self-healing capacity. The main treatment for end-stage joint pathology is currently total joint replacement, yet it does not afford long-term restauration [4].

Because of the limitations of two-dimensional (2D) *in vitro* systems (lack of differentiation, difficulties to reproduce the native cell environment), studies are always elaborating more complex and specific systems by combining cells, biomaterials, and biological or mechanical factors. Mesenchymal stem cells (MSCs) have now become a gold standard in regenerative medicine. These cells are capable of self-renewal, are multipotent, home to damaged tissues, and can modulate the surrounding immunity. MSCs are employed in many clinical trials involving for example treatments for graft versus host disease [5] or OA [6]. Although the most frequently used sources of MSCs are from adult origin like bone marrow or adipose tissue, perinatal sources such as Wharton's jelly in umbilical cord are potent alternatives as they do not request invasive procedures and offer larger quantities of cells with higher proliferation rate and greater immunomodulatory properties [7,8]. MSCs have also been proposed as good candidates for gene therapy and could be used as delivery vectors in many approaches [9].

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Therapeutic factors to treat bone and cartilage defects include growth factors and other agents (transcription factors, signaling molecules, anti-inflammatory agents), many of which have been tested as soluble (recombinant) molecules [10]. As recombinant factors only have a short half-life, their use may lead only to transient repair of the recipient tissues. Therefore, the use of therapeutic genes may promote more durable effects. The concept of gene therapy is based on the use of gene carriers (vectors) that may either have a nonviral origin (plasmids) or a viral one, the most classical of which being the adenoviral vectors, herpes simplex viral (HSV) vectors, retro-/lentiviral vectors, and recombinant adeno-associated viral (rAAV) vectors. Because of its advantages, gene therapy has already been used in bone [11] and cartilage regeneration [12].

Because cells need a three-dimensional (3D) environment to fully differentiate, and since the engineered tissue should be able to recruit autologous cells, a biomaterial has to be employed to repair cartilage or bone defects. Moreover, most biomaterials offer the possibility to design an implant of the desired size and shape which is also a requirement for surgical procedures. Synthetic materials like ceramic or tricalcium phosphate (TCP) are already often used to treat bone defects [13], but many studies consider now natural polysaccharides [14] or extracellular matrix [15] as valuable tools for tissue engineering. Indeed, such biomaterials can also guide cell differentiation towards a desired phenotype by their intrinsic properties, or even be used as a support for transfection vectors in order to raise their efficiency or lifetime.

Thanks to technological advances, there are even studies that now associate cells, biomaterials, and gene delivery vectors in bioinks to reconstitute 3D tissues [16]. This could allow to build quickly off-the-shelf implants usable for surgical procedures aiming to treat bone and cartilage injuries.

This minireview summarizes studies associating together MSCs, gene therapy and biomaterials in the field of cartilage and bone engineering.

2. CURRENT EVIDENCE *IN VITRO* AND *IN SITU*

A number of gene- and scaffold-based studies have been reported in the goal of reproducing bone and cartilage specifics in cell culture systems *in vitro* and in organ explant models *in situ* as a means to compensate for the loss of or to repair damaged tissues (**Figure 1**).

2.1. Formation of bone tissue *in vitro* and *in situ*

Growth factors have mostly been used as therapeutic candidates to activate the production of typical bone elements in these settings. Gonzalez-Fernandez *et al.* demonstrated that the association of plasmids encoding for the transforming growth factor beta (TGF-β3) or for the bone morphogenetic protein 2 (BMP-2) with hydroxyapatite nanoparticles in alginate hydrogels helped the plasmids to reach the cell nucleus of bone marrow-derived MSCs (BMMSCs) isolated from porcine femur [17]. In this case, the scaffold was not only a supportive matrix for the cells but also for the gene delivery vector thanks to hydroxyapatite nanoparticles which efficiently bind to plasmid DNA (pDNA). Moreover, MSCs transfected with both genes produced more sulfated glycosaminoglycan and type-II collagen, whereas MSCs transfected only with either TGF-β3 or BMP-2 had an enhanced calcium and type-X collagen deposition. Thus, a control on the cell fate can be achieved between osteogenic or chondrogenic pathways. One of the difficulties in bone engineering is to obtain a vascularized tissue to promote mineralization and rapid bone recovery. In this purpose, Jiang *et al.* demonstrated that rat BMMSCs isolated from the hind limb then co-transduced with BMP-2 and vascular endothelial growth factor 165 (VEGF165) lentiviral vectors and seeded on β-TCP scaffolds had an increased alkaline phosphatase (ALP) activity, thus indicating an early osteoblastic differentiation [18]. Here, the porous β-TCP scaffold was biocompatible and supported MSCs while it also afforded effective osteoconductivity.

As microRNAs (miRNAs) are able to modulate the levels of translational processes, they can also be transfected to direct cell differentiation towards a desired phenotype. In this goal, Shao and coworkers used nonviral vectors to express miR-122 in rat BMMSC sheets to improve osteogenesis in oral implants [19]. After complexation with titanium oxide implants, which are widely used in oral surgery, osteogenesis of the complex was raised by miR-122, with an increased expression of osteogenic markers like osteocalcin or type-I collagen. Alternatively, miRNA sponges directed against miR-140 and miR-240 have been reported to lead to the activation of osteogenesis and repression of osteoclastic activity in rat BMMSCs [20]. In the same paper, the authors demonstrated that gelatin sponges could be used to support MSCs and fill bone defects, thus evidencing the feasibility of transferring this technique from *in vitro* to *in vivo* settings.

2.2. Formation of cartilage tissue *in vitro* and *in situ*

Administration of extracellular cartilage matrix compounds themselves have been attempted to promote the production of cartilage elements. For instance, Guo *et al.* used multilayered films made of hyaluronic acid and DNA-lipid complexes to induce the expression of the cartilage oligomeric matrix protein (COMP) in MSCs [21]. Hyaluronic acid is highly represented in the extracellular matrix and was chosen as a biocompatible cation to form films with DNA-lipid complexes capable of transfecting MSCs. Such films could further be used to functionalize 3D surfaces, thus allowing *in vivo* translation of the findings. The cells were successfully transfected, showing higher chondrogenic gene expression levels with increased alcian blue staining, but also decreased ALP and alizarin red staining. Such functionalized scaffolds may be prepared in advance and then seeded with MSCs when needed, allowing them to differentiate and to regenerate the surrounding cartilage when transplanted.

Here also growth factors have been employed for cartilage tissue formation. Iwai *et al.* developed a cartilage defect model in osteochondral explants from porcine femurs where they seeded TGF-β3-transfected human MSCs isolated from bone marrow aspirates to monitor the extent of the lesions repair, showing an increased alcian blue coloration [22]. The explants represent the native tissue that

should be repaired and may be used as a 3D *in situ* model to analyze the ability of transfected MSCs to regenerate cartilage.

As the origin of the cells employed remains critical to the outcomes, Ikeda *et al.* monitored the differentiation of human synovial MSCs (a source of cells known to express less osteogenic genes than BMMSCs) upon transduction with a lentiviral vector to overexpress the insulin-like growth factor I (IGF-I) gene in a pellet culture model [23]. By using pellets, cells are allowed to synthetize their own matrix and the authors could follow the growing size of the pellets after 7 and 14 days, obtaining a 3D biomaterial that could be implanted into defects. The authors reported an elevation of type-II collagen production without expression of hypertrophic or osteogenic markers, thus showing that the matrix secreted was representative of hyaline cartilage.

2.3. Overview

In vitro studies focusing on the differentiation of MSCs and on their potential to overexpress bone or cartilage proteins have been useful as proof-of-principle of the combined gene therapy/tissue engineering concept but also to test new MSC sources (e.g. synovial MSCs) [23] and new biomaterials (e.g. hyaluronic acid-DNA complexes) [21], providing a basis to evaluations in clinically relevant experimental models *in vivo*. Studies show the importance of choosing properly the vector for gene transfer as it has to be clinically relevant for potential clinical trials. At the same time, biomaterials should be chosen carefully as they can be used only to support MSCs and fill a potential defect, but also have inner properties relevant for the gene delivery (*e.g.* interaction with pDNA) or tissue reparation (*e.g.* osteoconductivity).

3. CURRENT EVIDENCE *IN VIVO*

In vivo studies have been performed to provide a far more complete view on the results and constraints of the target tissues and organs, as evaluations are being performed in a natural three-dimensional environment in the presence of mechanical stress and of possible inflammation, and/or immune responses (**Figure 1**).

3.1. Treatment of bone defects

In 2010, Li *et al.* demonstrated that adenoviral transduction of rabbit BMMSCs isolated from the tibia with BMP-7 in association with a porous nano-hydroxyapatite/polyamide composite scaffold led to stronger ALP staining and and Col I immunostaining *in vitro* compared with nontransfected cells and accelerated bone formation in rabbit mandibular defects, although no difference was seen between animals treated with BMP-7-transfected MSC seeded scaffolds and the control groups (scaffold alone, MSC-seeded scaffold) after 16 weeks, indicating that other parameters may need to be taken into account for bone engineering (e.g. angiogenesis stimulation, cell seeding, biomaterial nature, etc.) [24]. The chosen biomaterial was made of hydroxyapatite which has a structure and composition close to native bone, and polyamide which has a structure close to collagen and strong mechanical properties. The alliance of hydroxyapatite and polyamide properties proved to be effective, with a bone regeneration obtained with the scaffold alone. However, mineralization was accelerated with BMP-7-transfected cells seeded on the scaffolds compared with nontransfected cells, and it was also faster with MSCs-seeded scaffolds compared with scaffolds alone, thus indicating the interest of using MSCs for bone regeneration.

BMP-2, as BMP-7, is known to promote osteogenesis. Castro-Govea *et al.* implanted *ex vivo*-BMP-2-transduced autologous BMMSCs isolated from humeral epiphyseal biopsy in dog mandibular defects, after seeding them in a demineralized bone matrix, which was shown to be an adapted support for MSCs in bone engineering as it stimulates bone induction [25]. Once showing that BMP-2 transduction enhances type-I collagen production and mineralization *in vitro*, the authors observed that the bone repair *in vivo* was almost complete after 10 weeks unlike nontransduced MSCs. Chang *et al.* also used BMP-2 as an osteoinducting factor in a more elaborated biomaterial [26]. These authors created defects of 4 cm diameter in the skull of miniature swine where they inserted a composite of gelatin, TCP ceramic and glutaraldehyde, surrounded by 2 layers of Pluronic F127 hydrogel containing BMP-2 transduced swine MSCs isolated from bone marrow aspirates. In this construction, the composite layer acted as a support for the new bone formation, while the external layers of hydrogels with BMP-2 producing MSCs stimulatedbone repair in the middle layer. The hydrogel was fully resorbed by surrounding cells after 2 weeks and the bone repair was improved after 6 months for transduced MSCs compared to nontransduced MSCs, with a thicker and stiffer well-mineralized bone. The authors concluded that the resorption speed of biomaterials should also be taken in account.

Loozen *et al.* further tested the behavior of biphasic calcium phosphate implants associated with alginate and BMP-2 expression plasmids in the presence (or absence) of caprine MSCs isolated from bone marrow aspirates in the iliac crest of goats [27]. MSCs were cultivated in an alginate hydrogel containing a plasmid encoding BMP-2 gene and dripped the gel on a porous scaffold made of hydroxyapatite and β-TCP. As a result, the presence of transfected MSCs led to higher BMP-2 expression and more efficient bone formation in the implant.

Other studies focused on transcription factors to stimulate osteogenesis. In 2009, Dong *et al.* modified rabbit MSCs isolated from the femur with the core binding factor α1, and showed an increase in the expression of osteopontin, osteocalcin, and type-I collagen *in vitro* associated with a higher ALP activity. They next used these transfected MSCs in association with acellular bone matrix, a material obtained from native bone which is ideal for bone tissue engineering thanks to its porosity and mechanical properties, and then inserted this construct into rabbit radial defects [28]. As a result, osteogenic marker expression and new bone formation was higher in the transfected-MSCs group, radiographies showed that the bone was healed and that animals recovered their motor behavior. Later, Wang *et al.* used osterix as an inducer of osteogenesis by implantation of human umbilical cord MSCs overexpressing this factor in a poly-D,L-lactide-co-glycolide (PLGA) scaffold in nude mice to enhance bone formation in regard of acellular or control-

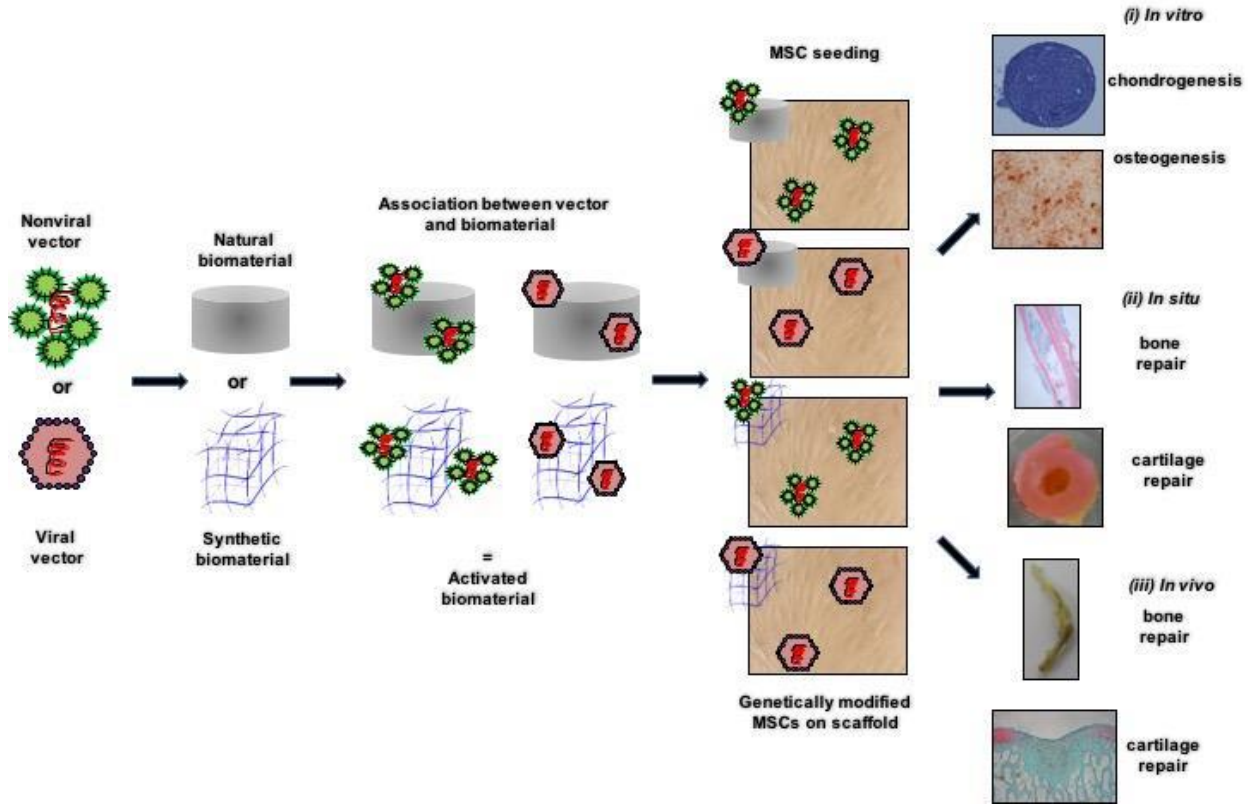

Figure 1. Combined use of MSCs, biomaterials and gene therapy for musculoskeletal engineering. Natural (*e.g.* hyaluronic acid) or synthetic (*e.g.* β-tricalcium phosphate) biomaterials may be activated by adjunction of nonviral (*e.g.* pDNA associated with hydroxyapatite nanoparticles or viral (*e.g.* recombinant adeno-associated virus) vectors carrying therapeutic genes to target MSCs, leading to *(i)* enhanced osteo/chondrogenesis *in vitro*, *(ii)* bone and cartilage repair in experimental explant defect models *in situ*, or *(iii)* bone and cartilage repair in clinically relevant defects in animal models *in vivo*.

transfected scaffold [29]. A scanning electron microscopy analysis showed that the PLGA scaffold was porous and suitable for bone tissue formation. The group containing osterix transfected MSCs in the PLGA scaffold showed a higher bone matrix component expression and an increased bone formation area.

As having a good blood supply is a major concern in bone formation, Raftery *et al.* developed a gene-activated scaffold made of collagen and hydroxyapatite with chitosan nanoparticles for the delivery of BMP-2 and VEGF [30]. Chitosan is biocompatible and biodegradable and was used here to carry the pDNA. The scaffold made of collagen could host both the chitosan nanoparticles and the MSCs, and fill the defect as well. When combined with hydroxyapatite, this scaffold had enhanced osteoinductive capacities by mimicking the bone matrix. These authors observed higher levels of osteogenesis in rat BMMSCs *in vitro* and bone formation after only 28 days in rat calvarial defects without the need for *ex vivo* culture. For comparison, the same level was reached in 8 weeks with only BMP-2 transfection, suggesting the importance of considering angiogenesis for the treatment of bone defects. Another path to enhance vascularization was explored by Cao *et al.* who transfected rabbit BMMSCs isolated from the tibia with angiopoietin-1 and seeded them on β-TCP scaffolds [31]. Artificial bone of β-TCP can fill small size defects efficiently by mimicking bone matrix but are not vascularized and so cannot be used in larger defects. Here, transfected cells showed increased proliferation and differentiation *in vitro*, and both bone formation and vascularization when implanted in rabbit radial defects, with a higher number of capillaries allowing a more efficient regeneration of bone and a mechanical resistance close of normal bone.

3D bioprinting proved an effective way to control the spatial distribution of cells and biomaterials. Indeed, Cunniffe *et al.* used a bioink made of alginate and nano-hydroxyapatite (the same biomaterial as in their previous works [17]) associated with TGF-β3 and BMP-2 plasmids mixed with pig BMMSCs isolated from the femur to promote osteogenesis [32]. These authors co-printed their biomaterial with a polycaprolactone mesh in order to enhance the mechanical resistance of the substitute. *In vitro*, they showed an enhanced matrix deposition and mineralization by the MSCs. The subcutaneous implantation

of MSC-seeded scaffolds in nude mice was successful, with an increase in matrix synthesis, mineralization and vascularization in MSC-seeded implants.

3.2. Treatment of cartilage defects

In 2011, Cao *et al.* obtained a repair of cartilage defects in a rabbit model by modifying rabbit MSCs isolated from bone marrow aspirates to overexpress the SRY-related high-mobility group (HMG) box 9 (Sox9), a key transcription factor involved in chondrogenesis. They seeded these BMMSCs on scaffolds built from poly(glycolic acid) (PGA), showing good biocompatibility and a capacity to be not fully degraded after 2 weeks. The transduced cells synthetized hyaline cartilage extracellular matrix compounds and expressed morechondrogenesis markers compared to cells without transduction both in the scaffolds cultured *in vitro* and after 12 weeks post-surgery in rabbit full-thickness cartilage defects [33].

The same year, in order to induce a higher chondrogenic differentiation, Yang and coworkers compared the transfection of human BMMSCs or human adipose-derived MSCs (ASCs) with a trio of transcription factors known to be master regulators of chondrogenesis (Sox5, Sox6, and Sox9) [34]. After encapsulation of MSC pellets in a fibrin hydrogel to mimic a 3D environment, MSC differentiation into mature chondrocytes was achieved both *in vitro* and *in vivo* upon implantation in nude mice, with production of type-II collagen and aggrecan, BMMSCs being more effective than ASCs with higher expression levels.

Another factor known to induce chondrogenesis is TGF-β1. Xia *et al.* transduced porcine MSCs isolated from bone marrow aspirates with TGF-β1 for 21 days to induce the growth factor production before seeding them on PGA scaffolds [35]. Then, the scaffolds were implanted subcutaneously into nude mice, and after 3 weeks a neocartilage was observed compared to control group. Moreover, the transfected group presented a neotissue with a higher volume and with a positive staining for sulfated proteoglycans and type-II collagen, and large amount of glycosaminoglycans (GAGs). He *et al.* chose another way to deliver this gene, and induced the differentiation of rat MSCs isolated from the femur with a pullulan-spermine vector inducing TGF-β1 expression in gelatin sponges to create a 3D implant [36]. These sponges where then implanted in an articular defect in the patellar groove of rats, allowing for effective cartilage repair, as shown by histological staining after 2 months.

In their study, Li *et al.* monitored the effect of TGF-β1 transfection in rabbit MSCs isolated from bone marrow aspirates via a macroporous PLGA sponge filled with fibrin using a complex of poly(ethylene oxide)-b-poly (L-lysine) (PEO-b-PLL) as low cytotoxic vector for pDNA-TGF-β1 delivery [37]. After 12 weeks of implantation in defects created in the patellar groove of rabbits, the construct showed a good GAGs and type-II collagen deposition as well as a higher expression of cartilage markers.

Interestingly, Ivkovic and coworkers used sheep bone marrow aspirates to repair cartilage [38]. The aim of the study was to develop a cost- and time preparation-effective biomaterial relative to *in vitro* engineered tissues. In their study, bone marrow has been allowed to coagulate in order to form a plug. The plug was implanted into partial-thickness defects formed on the medial condyle of sheep. The marrow was transduced with TGF-β1 gene before implantation. After 6 months, the cartilage repair was satisfying in transfected or untransfected plugs, but the TGF-β1-transfected group showed a higher production of type-II collagen and GAGs, showing that the MSCs present in the bone marrow aspirates were able to differentiate and synthetize new cartilage.

3.3. Overview

In vivo studies revealed the feasibility of combining gene therapy/tissue engineering to successfully achieve bone and cartilage repair in experimental, clinically relevant small and large animal models (**Figure 1**). Here, studies tried to employ appropriate vectors and biomaterials (as stated in the *in vitro* overview), but had to consider more parameters as *in vivo* experiments are the step preceding clinical trials. Biomaterials and vectors are especially adapted for their biocompatibility (*e.g.* chitosan nanoparticles associated with collagen and hydroxyapatite scaffold [30]) and their mechanical resistance (e.g. polycaprolactone mesh [32]) because cartilage and bone have to endure strong mechanical forces, especially when treating damages done to the tissue. Some works take also into account the cost of engineering techniques and try to employ more cost effective materials associated with easier preparation steps [38].

CONCLUSION

This minireview reveals that the association of biomaterials and gene therapy, in combination with MSCs shows a great potential for regenerative medicine in the field of bone and cartilage tissue engineering, with experiments done even in small and large animal models, often after preliminary *in vitro* tests (**Figure 1**). While MSCs have the ability to differentiate towards osteogenic or chondrogenic phenotypes and to regenerate tissue by synthetizing the appropriate matrix elements, biomaterials allow a 3D support for the cells until the defect is repaired, but can also act like a guide to stimulate the formation of new tissue (*e.g.* osteoconductivity of porous β-TCP scaffolds) [18]. Indeed, tissue regeneration can also be observed by implanting a cell-free biomaterial thanks to its mechanical properties and biocompatibility. However, there is still few studies trying to bring together all of these factors in order to achieve a rapid regeneration with a higher quality of recovered tissue.

There are recent works trying to answer to the most frequently encountered problems in bone therapy, like the lack of vascularization in engineered implants by transfecting MSCs with both angiogenic and osteogenic genes [30]. Another challenge for such a therapy is to successfully scale-up to be able to engineer implants of larger size, as defects in cartilage and bone can be of large size. However, the ability to obtain larger implants is tempered by the ability to form capillaries for bone to bring nutrients at the center of the implant. Additionally, it could be interesting to study further the use of gene therapy in combination with 3D printing as Cunniffe *et al.* did [32]. As achievements were accomplished in large animal models, more studies associating MSCs, gene therapy, and biomaterials

should be driven in the future, leading to clinical trials for bone and cartilage injuries.

The scope of application of such techniques could also be extended to other musculoskeletal tissues. Indeed, Gulotta *et al.* have demonstrated that rat BMMSCs transfected with the scleraxis transcription factor and embedded in a fibrin glue could positively affect rotator cuff tendon repair in a rat model [39].

In conclusion, it is encouraging to note that progress is being made by the combination of techniques developed in parallel, leading to more elaborated implants and thus refining the fabrication processes in bone and cartilage engineering.

LIST OF ABBREVIATIONS

2D: two-dimension; 3D: three-dimension; ALP: alkaline phosphatase; ASC: adipose-derived MSC; BMMSC: bone marrow-derived MSC; BMP: bone morphogenetic protein; COMP: cartilage oligomeric matrix protein; GAG: glycosaminoglycan; HMG: high-mobility group; HSV: herpes simplex virus; IGF: insulin-like growth factor; miRNA: microRNA; MSC: Mesenchymal stem cell; OA:osteoarthritis; pDNA: plasmid DNA; PEO-b-PLL: poly(ethylene oxide)-b-poly (L-lysine); PGA: poly(glycolic acid); PLGA: poly-D,L-lactide-co-glycolide; rAAV: recombinant adeno-associated virus; Sox: SRY-related HMG box; TCP: tricalcium phosphate; TGF: transforming growth factor; VEGF: vascular endothelial growth factor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Objectifs de la thèse

Les objectifs de ce travail de thèse ont été d'associer l'expertise reconnue en médecine régénérative humaine musculo-squelettique - utilisant notamment la thérapie génique - du « Zentrum für Experimentelle Orthopädie » (ZEO, Allemagne) dirigé par le Professeur Magali Cucchiarini, à celle du groupe « ingénierie vasculaire » dirigé par le Professeur Patrick Menu de l'UMR IMoPA (France), afin de constituer des biomatériaux hydrides à base de CSM utilisables en ingénierie musculo-squelettique.

Dans un premier temps, le travail s'est appuyé sur les compétences du groupe ingéniérie vasculaire et s'est concentré sur la formation de vaisseaux de faible diamètre pouvant présenter un intérêt dans l'ingéniérie musculosquelettique notamment osseuse. En effet, dans un contexte de fractures de grande taille nécessitant de créer des implants osseux importants, la vascularisation doit être prise en compte. La différenciation des CSM-GW vers un phénotype endothélial (constituant l'intima vascualaire) étant déjà maîtrisée par notre groupe, la différenciation *in vitro* des CSM-GW cultivées en monocouche sur supports standards vers un phénotype musculaire lisse (constituant la media vasculaire) a été testée en présence de TGF-ß1 ou d'acide ascorbique (*cf.* Article n°3). Les résultats obtenus ont mis en évidence que pour favoriser une différenciation cellulaire plus complète et plus durable, il était nécessaire de développer un modèle de différenciation en 3 dimensions en s'appuyant sur les compétences du ZEO.

Le ZEO réalise le transfert de gènes thérapeutiques par des virus adéno-associés recombinants (rAAV). Les rAAV sont déjà clonés et préparés en routine au ZEO, notamment des vecteurs porteurs de gènes thérapeutiques spécifiques du cartilage (rAAV-FLAG-h*sox9* portant le facteur de transcription SOX9). Nous avons donc mis au point un modèle de biomatériau hybride en nous focalisant sur la différenciation chondrogénique (expertise du ZEO) de CSM-GW (cellules couramment employées par IMoPA). Ce modèle a pour but d'associer les bénéfices des CSM-GW, de la MEC-GW et de la thérapie génique (présentés dans l'introduction) dans un système de culture en 3 dimensions. Les résultats de ces expériences avaient pour but d'identifier l'association optimale entre les CSM-GW, la MEC-GW et les rAAV ainsi que les conditions favorables à la chondrogenèse dans notre modèle (*cf.* Article n°4), ceci dans le but de constituer des implants capables de régénérer des lésions du cartilage. L'objectif à plus long terme de ce travail étant également d'étendre le modèle aux lésions osseuses.

Partie 1 : Différenciation vasculaire de CSM-GW

Publication n°3 : Article scientifique

Résumé en français

Le transforming growth factor-ß1 et l'acide ascorbique permettent de différencier les CSM-GW vers un phénotype musculaire lisse.

Benjamin Mesure, Sophie Huber-Villaume, Patrick Menu et Émilie Velot.

Publié dans Bio-Medical Materials and Engineering n°28 en 2017.

Au début de la thèse, ce travail s'est inscrit dans la continuité de la thématique du groupe « Ingénierie Vasculaire ». Notre groupe a déjà démontré plusieurs fois la faisabilité de la différenciation de CSM-GW vers un phénotype endothélial, aussi bien sur des films multicouches de polyélectrolytes [19] que sur des lames de verre recouvertes par un dépôt de MEC de GW [37]. Nous nous sommes donc naturellement tournées vers la différenciation de CSM-GW vers un phénotype de CML, l'autre principal type cellulaire rencontré dans les vaisseaux sanguins. Dans cette étude nous avons étudié l'effet de l'ajout de transforming growth factor-ß1 (TGF-ß1) ou d'acide ascorbique (AA) dans le milieu de culture de CSM-GW sur cette différenciation, suivie par Western Blot pour l'expression de marqueurs caractéristiques des CML. Après 12 jours de culture, l'expression de marqueurs précoces (tels que l'alpha actine du muscle lisse ou la protéine SM22) et intermédiaires (tels que la calponine ou la caldesmone) de la différenciation des CML a été mise en évidence, notamment dans les conditions présentant les plus fortes concentrations en TGF-ß1 et AA. En revanche, l'expression du marqueur caractéristique des CML matures, la chaîne lourde de la myosine du muscle lisse, n'a pas été constatée. Cela nous a poussé à conclure que les conditions dans lesquelles était réalisée cette différenciation (2 dimensions *in vitro*), ne permettaient pas aux cellules d'acquérir pleinement le phénotype souhaité. Cela a donc orienté notre réflexion vers l'élaboration de biomatériaux en 3 dimensions impliquant des facteurs de différenciation plus efficaces (utilisation de la thérapie génique), en dialogue avec le ZEO.

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Transforming growth factor-beta 1 or ascorbic acid are able to differentiate Wharton's jelly mesenchymal stem cells towards a smooth muscle phenotype

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Abstract. Wharton's jelly mesenchymal stem cells (WJ-MSCs) are widely used in tissue engineering. In vascular engineering, the ability to obtain a vessel replacement with contractile smooth muscle cells (SMC) is a key factor. In this work, we demonstrated that WJ-MSCs differentiate towards a SMC phenotype with various stimulations *in vitro* and that the modification of redox state could be involved. WJ-MSCs were isolated from umbilical cords. After their expansion, the cells were stimulated with ascorbic acid (AA, 300 μM) or transforming growth factor (TGF)-β1 (1 to 5 ng/mL). SMC markers were analyzed by Western blot. Modification of redox state was evaluated by reactive oxygen species (ROS) production and glutathione (GSH) content measurements. TGF-β1 or AA-stimulated WJ-MSCs express early and intermediate SMC markers. TGF-β1 (5 ng/mL) modifies the redox state by a ROS production and a GSH content drop, while AA has no effect. This work showed that TGF-β1 and AA are effective SMC phenotype inducers to differentiate WJ-MSCs.

Keywords: TGF-β1, ascorbic acid, Wharton's jelly-MSCs, smooth muscle cells, oxidative stress

1. Introduction

Vascular engineering is based on the development of substitutes mimicking natural vessels and can involve the combination of cells and biomaterials. Human vessel are made of cells such as endothelial cells and smooth muscle cells (SMCs), which are hard to expand and dedifferentiate *in vitro* [1]. Current researches in vascular engineering focus on mesenchymal stem cells (MSCs) as alternative to vascular primary cells because of their multipotency, notably those from the Wharton's jelly (WJ) of the umbilical cord. Compared to their adult counterparts, WJ-MSCs have a non-invasive isolation and are present in a large quantity. They are also more proliferative and immunosuppressive [2]. The differentiation of WJ-MSCs towards an endothelial phenotype has been achieved [3], however their differentiation into SMCs remains to be carried out. SMC differentiation has already been obtained with other sources

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of MSCs, such as bone marrow, after transforming growth factor-beta 1 (TGF-β1) or ascorbic acid (AA) stimulations [4]. Vascular SMC phenotype can be assessed by the expression of specific proteins: smooth muscle α-actin (SMA) and SM22 (early markers); calponin (CALP) and caldesmon (CALD) (intermediate markers); and smooth muscle myosin heavy chain (SM-MHC) (late marker of mature contractile SMCs) [5]. Oxidative stress due to reactive oxygen species (ROS) production and gluthatione (GSH) drop [6], or signaling pathways such as Smad 2/3 [7] can be involved in TGF-β1-induced specific SMC markers expression, while AA, a well-known antioxidant, can induce these specific markers by reducing the oxidized phospholipids [8].

The aim of our work is to show that TGF-β1 or AA are effective SMC phenotype inducers to differentiate WJ-MSCs.

2. Material and methods

2.1. Cell isolation, stimulation and phenotype evaluation

WJ-MSCs were isolated, expanded and characterized as previously described [9]. At the 4th passage, WJ-MSCs were seeded at a density of 1000 cells/cm^2 and grown to 60–70% of confluency. Then, the cells were cultured in DMEM low glucose with 2% FBS and 2 mM glutamine. The condition with medium alone was used as control. To trigger differentiation towards SMCs, the cells were stimulated with TGF-β1 (1, 2 and 5 ng/mL) or AA (300 μM). After 12 days of stimulation, smooth muscle markers were analysed by Western blot as previously described [3]. The primary antibodies used were: anti-SMA (Dako, 1/32000), anti-SM22 (Sigma, 1/10000), anti-calponin (Dako, 1/2500), anti-caldesmon (Millipore, 1/15000), anti-SM-MHC (Dako, 1/2500) and anti-β-tubulin (Santa Cruz, 1/1000) as reference.

2.2. ROS production detection and GSH content measurement

WJ-MSCs were stimulated with 5 ng/mL of TGF-β1 or 300 μM of AA for various times up to 48 h. Cells were loaded with 50 μM DCFDA (2′,7′-dichlorofluorescin diacetate) during 15 min at 37°C, washed with DMEM and ROS were detected by fluorescence microscopy. After stimulation, GSH content was extracted and quantified as previously described [10].

2.3. Statistical analysis

Results are expressed by mean $\pm$ SD ($n = 3$). Differences between control and stimulated cells were analysed by Student's t-test and were considered significant with $p < 0.05$.

3. Results

3.1. Expression of SMC specific markers

After 12 days of stimulation, the late marker SM-MHC wasn't induced but the expression of early (SMA, SM22) and intermediate markers (CALP, CALD) was increased, mainly in the presence of high concentrations of TGF-β1 or 300 μM AA (Fig. 1). As the most efficient conditions, the highest TGF-β1 concentration and AA were chosen for further investigations.

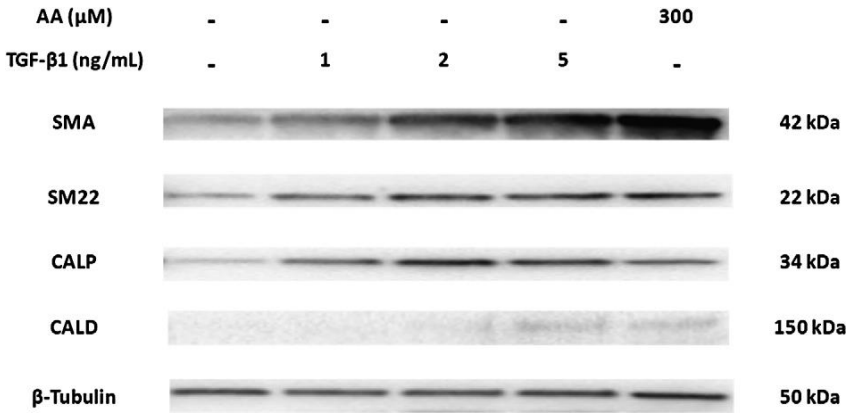

Fig. 1. Expression of SMC markers by TGF-β1 or AA stimulated WJ-MSCs. WJ-MSCs were stimulated by transforming growth factor (TGF)-β1 or ascorbic acid (AA) for 12 days. The expression of smooth muscle cell (SMC) markers was then analysed by Western blot. (Representative picture of three independent experiments; SMA: smooth muscle alpha actin; CALP: Calponin; CALD: Caldesmon.)

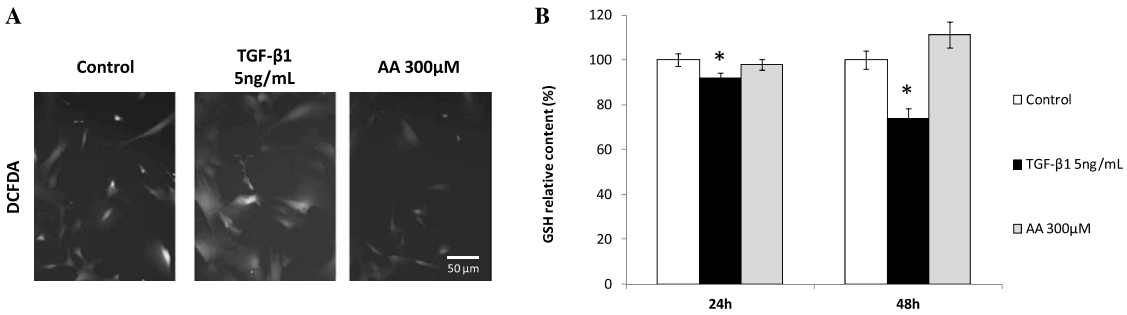

Fig. 2. ROS production (A) and GSH content drop (B) in TGF-β1 or AA stimulated-WJ-MSCs. (A) WJ-MSCs were stimulated by transforming growth factor (TGF)-β1 or ascorbic acid (AA) for 24 h and dyed with 2′,7′-dichlorofluorescin diacetate (DCFDA). The fluorescent signal supports the presence of reactive oxygen species (ROS). (Representative picture of three independent experiments.) (B) Glutathione (GSH) levels are expressed in percentage obtained from WJ-MSCs stimulated by TGF-β1 or AA during 24 h and 48 h related to control. (Means $\pm$ SD, $n = 3$, $* : p < 0.05$, relative to control.)

3.2. Modification of cellular redox state

ROS production was observed in TGF-β1-stimulated WJ-MSCs (5 ng/mL) after 2 hours (Data not shown). This phenomenon persisted up to 24 h (Fig. 2(A)). No ROS were detected after 48 h of stimulation (Data not shown). This oxidative stress was correlated with a GSH content drop. This effect was more important after 48 h of stimulation (Fig. 2(B)). No ROS production and GSH content modification were visible in AA stimulated-WJ-MSCs, whatever the time of stimulation (Fig. 2(A) and (B)).

4. Discussion

TGF-β1 or AA induce the expression of SMC markers in WJ-MSCs but the differentiation is incomplete as the expression of the mature marker SM-MHC was not detected. These results matched with

those performed with bone marrow (BM)-MSCs [4]. To complete this differentiation, the next step will consist in culturing WJ-MSCs into a 3D architecture and under mechanical stress to express mature contractile SMC markers. TGF-β1 and AA could also be associated as they have an additive effect on SMC markers in BM-MSCs [4].

TGF-β1 induces ROS production and GSH content drop in WJ-MSCs. These results were already obtained with others cells, such as SMCs or endothelial cells [7,11]. This modification of the redox state could be involved in SMC markers expression, as described in aortic SMCs [7]. Catalase or N-acetylcystein could be used to determine the implication of TGF-β1 induced-oxidative stress in this effect. As AA has no effect on redox state in WJ-MSCs, the induction of SMC markers expression after AA stimulation could be induced by another signaling pathway. Indeed, reduction of oxidized phospholipids by AA could counteract the inhibition of SMC contractile markers expression [8].

Conflict of interest

The authors have no conflict of interest to report.

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Partie 2 : Différenciation chondrogénique de CSM-GW

Publication n°4 : Article scientifique

Résumé en français

Influence de la matrice extracellulaire du cordon ombilical sur le potentiel chondrogénique des cellules souches mésenchymateuses humaines de la gelée de Wharton génétiquement modifiées pour surexprimer SOX9 via un transfert de gène par rAAV

Benjamin Mesure, Patrick Menu, Jagadeesh K. Venkatesan, Gertrud Schmitt, Émilie Velot, and Magali Cucchiarini.

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La greffe de biosubstituts comportant des cellules souches génétiquement modifiées dans les sites de lésions cartilagineuses est une approche de choix pour renforcer les processus de régénération dans de telles lésions, en particulier en utilisant les CSM-GW qui ont un potentiel chondrogénique fort et qui sont plus primitives que les CSM présentes dans la moelle osseuse des individus adultes. Nos objectifs de travail pour cet article étaient de démontrer que les CSM-GW pouvaient être transduites par rAAV et surexprimer SOX9, un facteur de transcription hautement chondrogène, afin d'induire une différenciation chondrogénique par rapport au traitement témoin (gène rapporteur *lacZ*). Basées sur les travaux antérieurs d'IMoPA concernant les MEC naturelles, les transductions ont été effectuées dans un modèle de culture tridimensionnel sous forme de pellets en présence ou non d'une solution de matrice extracellulaire naturelle issue de la gelée de Wharton (MEC-GW). Les CSM-GW ont été traduites avec succès et ont exprimé le gène *lacZ* jusqu'à 21 jours de culture. L'expression de *lacZ* a été augmentée en présence de MEC-GW. La surexpression de SOX9 médiée par rAAV a entraîné une différenciation vers un phénotype chondrogénique des CSM-GW. Ceci a été démontré par l'augmentation de la synthèse de protéoglycanes et du collagène de type II. De plus, cette augmentation est plus importante en présence de MEC-GW. Les résultats obtenus montrent l'intérêt d'utiliser une telle stratégie basée sur les cellules souches, la MEC-GW et les rAAV dans de futurs traitements pour les lésions du cartilage articulaire *in vivo*.

Influence of umbilical cord extracellular matrix on the chondrogenic potential of human Wharton's jelly mesenchymal stem cells genetically modified to overexpress SOX9 via rAAV-mediated gene transfer

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Running title: hWJ-MSC chondrogenesis by rAAV SOX9 in WJ-ECM

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Abstract

The implantation of genetically modified progenitor cells in sites of cartilage injury is a valuable approach to strengthen the reparative processes in such lesions, especially when using the safe, chondrogenically potent human mesenchymal stem cells of the Wharton's jelly (hWJ-MSCs) that are more primitive than those present in the bone marrow of adult individuals. The goal of the present study was to evidence whether human WJ-MSCs are amenable to gene transfer via clinically adapted recombinant adeno-associated viral (rAAV) vectors and can overexpress SOX9, a highly chondrogenic transcription factor, in order to trigger the chondrogenic commitment of such cells *versus* control (reporter *lacZ* gene) treatment in their natural, extracellular matrix (WJ-ECM). Sustained *lacZ* expression was achieved in hWJ-MSCs, especially when maintained in their WJ-ECM. The rAAV-mediated *sox9* overexpression further increased the chondrogenic activities in hWJ-MSCs most particularly in their three-dimensional WJ-ECM. The present findings show the value of using such a cell- and rAAV-based strategy in future setups for the treatment of articular cartilage defects *in vivo*.

Keywords: cartilage repair, hWJ-MSCs, WJ-ECM, rAAV, SOX9, chondrogenesis

Introduction

The articular cartilage is an essential tissue that permits a smooth gliding of the articulations but in adults, as a result of its lack of vascularization, it is not capable of fully regenerating in response to traumatic or degenerative injury, rather leading to the formation of a poorly functional fibrocartilaginous repair tissue in cartilage lesions (type-I *versus* type-II collagen and proteoglycans typical of the hyaline cartilage) [Buckwalter & Mankin 1998 487; Madry 2011 669]. Classical surgical treatments, even those based on marrow stimulation techniques, are still not capable of providing effective options to permanently reconstitute the hyaline cartilage surface that would reliably support physiological loads in patients [Smith 2005 445; Madry 2011 669]. Administration of adult progenitor cells originating from the bone marrow (bone marrow-derived mesenchymal stem cells - BM-MSCs) may be a well adapted strategy to increase the processes of chondroregeneration in sites of cartilage damage [Bruder 1994 283; Caterson 2001 E1; Johnstone 2001 915; Orth 2014 1]. Nevertheless, clinical attempts at injecting such cells in cartilage lesions have not led thus far to the reproduction of the hyaline cartilage, promoting instead the generation of a poorly integrative fibrocartilage [Wakitani 2004 595; Skowroński & Rutka 2013 195]. In this regard, the use of cells that are more primitive than adult BM-MSCs, and still display safe, clinically adapted profiles such as those present in the Wharton's jelly (WJ-MSCs) [Chen 2014 e98565; El Omar 2014 523], may provide strong alternatives for applications aiming at enhanced cartilage repair [Orth 2014 1]. WJ-MSCs that have been reported for their ability to commit towards the chondrogenic phenotype [Wang 2004 1330; Baksh 2007 1384; Troyer 2008 591; Gauthaman 2012 235; Orth 2014 1] are being currently tested in patients with orthopaedic lesions [Sadlik 2018 e57], yet

their use may be impeded by the large number of cells needed for implantation and by the low numbers of which that effectively generate an adequate cartilage repair tissue devoid of hypertrophic features [Prockop 2009 939].

Gene therapy based on the direct administration of potent chondroreparative genes in MSCs may provide convenient tools to activate the processes controlling the healing of injured articular cartilage [Evans 2000 s214; Yoo 2000 s164; Cucchiarini & Madry 2005 1495; Cucchiarini & Madry 2010 135; Frisch 2015 121]. Especially recombinant adeno-associated viral (rAAV) vectors are well suited systems as they can highly and persistently transduce mesenchymal stem cells (MSCs) (~ 100% efficacy for at least 3 weeks *in vitro*) without consequences on their differentiation potency [Pagnotto 2007 804; Cucchiarini 2011 1921; Venkatesan 2012 22]. Several therapeutic genes have been investigated for their direct ability to trigger the chondroreparative activities of human BM-MSCs (hBM-MSCs) (cartilage oligomeric matrix protein - COMP, transforming growth factor beta - TGF-β, bone morphogenetic proteins - BMPs, basic fibroblast growth factor - FGF-2, insulin-like growth factor I - IGF-I, zinc-finger protein 145 - ZNF145, Indian hedgehog - Ihh, small interfering RNA or siRNA against the osteogenic Cbfa-1 transcription factor, microRNAs such as miR-140 and miR-410) [Pagnotto 2007 804; Cucchiarini 2011 1921; Liu 2011 2711; Haleem-Smith 2012 1245; Jeon 2012 4413; Steinert 2012 R168; Frisch 2014a 103; Frisch 2014b 1050; Zhang 2017 136 PMID 28123640; Hossein PMID 29040159]. The transcription factor SOX9, a member of the sex-determining region Y-type high-mobility group box (SOX) family, may be a particularly suited therapeutic option as it plays key roles in cartilage formation and chondrocyte differentiation [Bi 1999 85]. While a number of studies showed the benefits of *sox* gene transfer in hBM-MSCs [Jeon 2012 4413; Venkatesan 2012 22], the feasibility of overexpressing such

sequences in hWJ-MSCs has been reported only by Wang *et al*. [Wang 2014 279 PMID 24652327] who employed nonviral gene transfer, achieving high gene transfer efficiencies but only for a relatively short period of time (80% for 2 days), while rAAV vectors allow to persistently reach such levels for months to years [Xiao 1996 8098], making them more adapted for sustainable clinical applications [Cucchiarini & Madry 2005 1495; Evans & Huard 2015 234; Cucchiarini 2016 495].

The goal of the present study was therefore to examine whether hWJ-MSCs are permissive to rAAV and can overexpress *sox9* via this vector class as a means to activate the metabolic and chondrogenic processes in such cells *in vitro* relative to control (reporter *lacZ* gene) treatment, especially when maintained in their natural extracellular matrix (WJ-ECM) [Dan 2017 227; Dan 2017 s95]. For the first time, to our best knowledge, we provide evidence showing that rAAV can mediate effective, prolonged gene overexpression in hWJ-MSCs *in vitro*. Moreover, hWJ-MSCs genetically modified to overexpress SOX9 via rAAV-mediated gene transfer showed enhanced levels of matrix deposition, and chondrogenic differentiaton in the cells relative to control treatment. These findings provide a basis to further test the current cell- and rAAV-based approach as a promising tool to treat articular cartilage defects *in vivo*.

Materials and Methods

Reagents

All reagents were from Sigma (Munich, Germany) unless otherwise indicated. Recombinant FGF-2 was purchased at Peprotech (Hamburg, Germany). Recombinant TGF-β3 was from R&D Systems (Wiesbaden, Germany). Dimethylmethylene blue was from Serva (Heidelberg, Germany). The anti-β-gal (GAL-13) antibody was from Sigma, and the anti-type-II collagen (II-II6B3) antibody from the NIH Hybridoma Bank (University of Iowa, Ames, USA). Secondary biotinylated secondary antibodies and the ABC kit were from Vector Laboratories (Grünberg, Germany).

Cell culture

Human umbilical cords (~ 20 cm) were obtained from after full-term births (n = 3). The study was approved by the University Hospital Center (CHU) of Nancy (TCG/11/R11). Human Wharton's jelly MSCs (hWJ-MSCs) were isolated and expanded in culture according to standard protocols [Rammal 2013 299; Mesure 2017 S101]. Briefly, umbilical cord vessels were removed from cord segments and exposed mesenchymal tissue was cut into small pieces (1-2 mm^3) that were treated for 18 h with collagenase type 2 (1 IU/ml) (ThermoFisher, Walthma, USA) in Dulbecco's modified Eagle's medium (DMEM) at 37°C with 5% CO_2 followed by enzyme inactivation using decomplemented fetal bovine serum (FBS). hWJ-MSCs were resuspended and maintained in alpha Minimal Eagle Medium (α-MEM) (Lonza, Basel, Switzerland) containing 10% FBS with 100 IU/ml penicillin/streptomycin (growth medium) and 2 mM glutamine at 37°C in a humidified atmosphere of 5% CO_2 with medium change every

2 days. Human WJ-MSCs were analysed by flow cytometry for expression of stem cell surface markers ($CD73^+$, $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$) [Rammal 2013 299; El Omar 2016 342]. All experiments were performed with cells at no more than passage 4.

Preparation of extracellular matrix from Wharton's jelly

Wharton's jelly extracellular matrix (WJ-ECM) was mechanically obtained from human umbilical cords (n = 3) using an extraction method adapted from work based on an enzymatic technique [Dan 2017 227; Dan 2017 S95]. Briefly, the cords were rinsed in Hank's balanced salt solution, the umbilical cord vessels were removed from cord segments, and exposed mesenchymal tissue was cut into small pieces (1-2 mm^3). The pieces were washed three times in phosphate buffered saline x1 (PBSx1) and centrifuged for 5 min at 300xg. The tissue was then teared for at least 20 min using an Ultra-Turrax disperser (IKA®, Staufen, Germany). The solution obtained was frozen at -80°C and lyophilized. The WJ-ECM was then resuspended in PBSx1 at a concentration of 20 mg/ml and centrifuged for 10 min at 1,600xg to separate the soluble matrix from the remaining solid pieces.

Plasmids and rAAV vectors

The constructs were derived from pSSV9, an AAV-2 genomic clone [Samulski 1987 3096; Samulski 1989 3822]. rAAV-*lacZ* carries the *lacZ* gene for *E. coli* β-galactosidase (β-gal) and rAAV-FLAG-h*sox9* a FLAG-tagged human *sox9* cDNA both under the control of the cytomegalovirus immediate-early (CMV-IE) promoter [Cucchiarini 2011 1921; Venkatesan 2012 22; Rey-Rico 2016 20600; Venkatesan 2017 22]. The vectors were packaged as conventional (not self-complementary) vectors using a helper-free,

two-plasmid transfection system in the 293 packaging cell line (an adenovirus-transformed human embryonic kidney cell line) with the packaging plasmid pXX2 and the Adenovirus helper plasmid pXX6 as previously described [Venkatesan 2012 22; Rey-Rico 2016 20600; Venkatesan 2017 22]. The vector preparations were purified by dialysis and titered by real-time PCR [Cucchiarini 2011 1921; Venkatesan 2012 22; Rey-Rico 2016 20600; Venkatesan 2017 22], averaging 10^{10} transgene copies/ml (viral particles to functional vectors = 500/1).

rAAV-mediated gene transfer

Aggregate cultures were allowed to form spontaneously in growth medium in the presence or absence of WJ-ECM (WJ-ECM:DMEM at 1:1). Aggregate cultures of hWJ-MSCs (2 x 10^5 cells per aggregate) were transferred in defined chondrogenic medium (high-glucose DMEM 4.5 g/l, penicillin/streptomycin, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.35 μg/ml linoleic acid, 1.25 μg/ml bovine serum albumin, 1 mM sodium pyruvate, 37.5 μg/ml ascorbate 2-phosphate, 10^{-7} M dexamethasone, 10 ng/ml TGF-β3) for transduction with rAAV (40 μl vector, i.e. 8 x 10^5 functional recombinant viral particles, i.e. MOI = 4) [Cucchiarini 2011 1921; Venkatesan 2012 22]. Cultures were then kept in defined chondrogenic medium for up to 21 days with medium change every 2 days [Cucchiarini 2011 1921; Venkatesan 2012 22].

Transgene expression

Expression of *lacZ* was monitored by immunohistochemistry on paraffin-embedded sections of aggregates (5 μm), respectively, using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with diaminobenzidine (DAB) as the chromogen [Venkatesan 2012 22; Venkatesan 2017 22]. Samples were examined by light microscopy (BX 45; Olympus, Hamburg, Germany) [Venkatesan 2012 22; Venkatesan 2017 22]. To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.

Biochemical assays

Cultures were harvested and digested with papain [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22]. The proteoglycan contents were determined by binding to dimethylmethylene blue dye, [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22]. Data were normalized to total cellular proteins using a protein assay (Pierce Thermo Scientific, Fisher Scientific, Schwerte, Germany). All measurements were performed with a GENios spectrophotometer/fluorometer.

Histological, immunocytochemical, and immunohistochemical analyses

Aggregate cultures were harvested and fixed in 4% formalin, dehydrated in graded alcohols, embedded in paraffin, and sectioned (5 μm). Sections were stained with toluidine blue (matrix proteoglycans) [Cucchiarini 2011 1921; Venkatesan 2012 22; Rey-Rico 2016 20600; Venkatesan 2017 22]. Expression of type-II collagen was detected by immunohistochemistry using specific primary antibodies, biotinylated

secondary antibodies, and the ABC method with DAB [Cucchiarini 2011 1921; Venkatesan 2012 22; Rey-Rico 2016 20600; Venkatesan 2017 22]. To control for secondary immunoglobulins, sections were processed with omission of the primary antibody. Samples were examined under light microscopy (BX 45; Olympus).

Real-time reverse transcription polymerase chain reaction analyses

Total cellular RNA was extracted from the cultures using the RNeasy Protect Mini Kit with an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). RNA was eluted in 30 μl RNase-free water. Reverse transcription was carried out with 8 μl eluate using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Applied Science) [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22]. An aliquot of the cDNA product (2 μl) was amplified with real-time PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) on an Mx3000P QPCR operator system (Stratagene) as follows: initial incubation (95°C, 10 min), amplification for 55 cycles (denaturation at 95°C, 30 sec; annealing at 55°C, 1 min; extension at 72°C, 30 sec), denaturation (95°C, 1 min), and final incubation (55°C, 30 sec) [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22]. The primers (Invitrogen, Darmstadt, Germany) used were SOX9 (chondrogenic maker) (forward, 5′-ACACACAGCTCACTCGACCTTG-3′; reverse, 5′-GGGAATTCTGGTTGGTCCTCT-3′), aggrecan (ACAN; chondrogenic maker) (forward 5’-GAGATGGAGGGTGAGGTC-3’; reverse 5’-ACGCTGCCTCG GGCTTC- 3’), type-II collagen (COL2A1; chondrogenic maker) (forward, 5′-GGACTTTTCTCCCCTCTCT-3′; reverse, 5′-GACCCGAAGGTCTTACAGGA-3′), type-X collagen (COL10A1; marker of hypertrophy) (forward 5’-CCCTCTTGTTAGTGCCAACC-3’; reverse 5’-AGATTCCAGTCCTTGGGTCA-3’), and glyceraldehyde-3-phosphate dehydrogenase

(housekeeping gene and internal control) (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′) (all 150 nM final concentration) [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22]. Control conditions included reactions using water and nonreverse-transcribed mRNA. Specificity of the products was confirmed by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) value for each gene of interest was measured for each amplified sample using MxPro QPCR software (Stratagene), and values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression by using the $2^{-\Delta\Delta Ct}$ method, as described previously [Cucchiarini 2011 1921; Venkatesan 2012 22; Venkatesan 2017 22].

Results

Effective rAAV-mediated transgene expression in human Wharton's jelly-derived mesenchymal stem cells via rAAV-mediated gene transfer using Wharton's jelly extracellular matrix

Human Wharton's jelly MSCs (hWJ-MSCs) were first transduced with the rAAV-*lacZ* vectors in the presence of Wharton's jelly extracellular matrix (WJ-ECM) to examine the feasibility of overexpressing transgene in the cells relative to conditions in the absence of WJ-ECM (**Figure 1**).

Highly efficient transgene (*lacZ*) expression was detected in hWJ-MSC aggregates when applying the rAAV-*lacZ* vector over a prolonged period of time known to support hMSC chondrogenesis (21 days) [Cucchiarini 2011 1921; Venkatesan 2012 22]. The presence of WJ-ECM in the cultures seems to improve the transduction. As previously shown in hBM-MSCs the *lacZ* signal became weaker at day 21 [Cucchiarini 2011 1921; Venkatesan 2012 22].

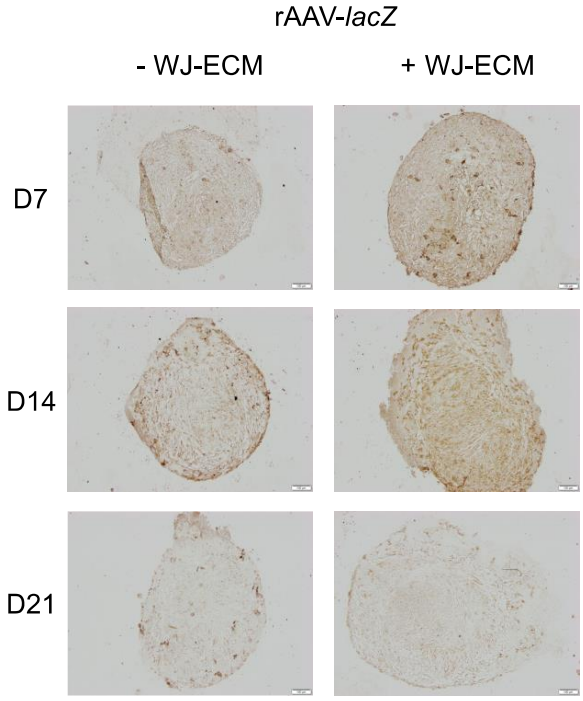

Figure 1. Detection of *lacZ* transgene expression in rAAV-transduced hWJ-MSC aggregates in the presence or absence of Wharton's jelly extracellular matrix. Cells in aggregate cultures were transduced with rAAV-*lacZ* (40 μl each vector, MOI = 4) without or with Wharton's jelly extracellular matrix (-/+ WJ-ECM, respectively). The aggregates were maintained in culture for 21 days and the cultures were processed to detect *lacZ* expression by immunohistochemistry as described in the Materials and Methods (magnification x100; scale bar = 100 μm; all representative data).

Biological and chondrogenic effects of rAAV-mediated SOX9 overexpression in human Wharton's jelly-derived mesenchymal stem cells using Wharton's jelly extracellular matrix

Chondrogenically-induced aggregate cultures of hWJ-MSCs were next transduced with rAAV-FLAG-hs*ox9* to analyze the effects of SOX9 overexpression upon the biological and chondrogenic activities of the cells in the presence of WJ-ECM *versus* control (rAAV-*lacZ*) treatment, and conditions in the absence of extracellular matrix. As we already evidenced that rAAV gene transfer had neither toxic nor advantageous effect on hMSC chondrogenesis [Cucchiarini 2011 1921; Venkatesan 2012 22], a test condition without vector treatment was not further included in the present analyses.

Chondrogenic differentiation was successfully achieved in the hWJ-MSC aggregates days especially in the presence of the s*ox9* vector relative to *lacZ* treatment as noted by a stronger toluidine blue staining (**Figure 2**) and type-II collagen immunostaining (**Figure 3**). The presence of WJ-ECM in the cultures seems to improve proteoglycan synthesis and chondrogenic activity (**Figure 2** and **Figure 3**). Production of proteoglycans was achieved in all tested conditions as showed by biochemical assay **(Figure 4)**.

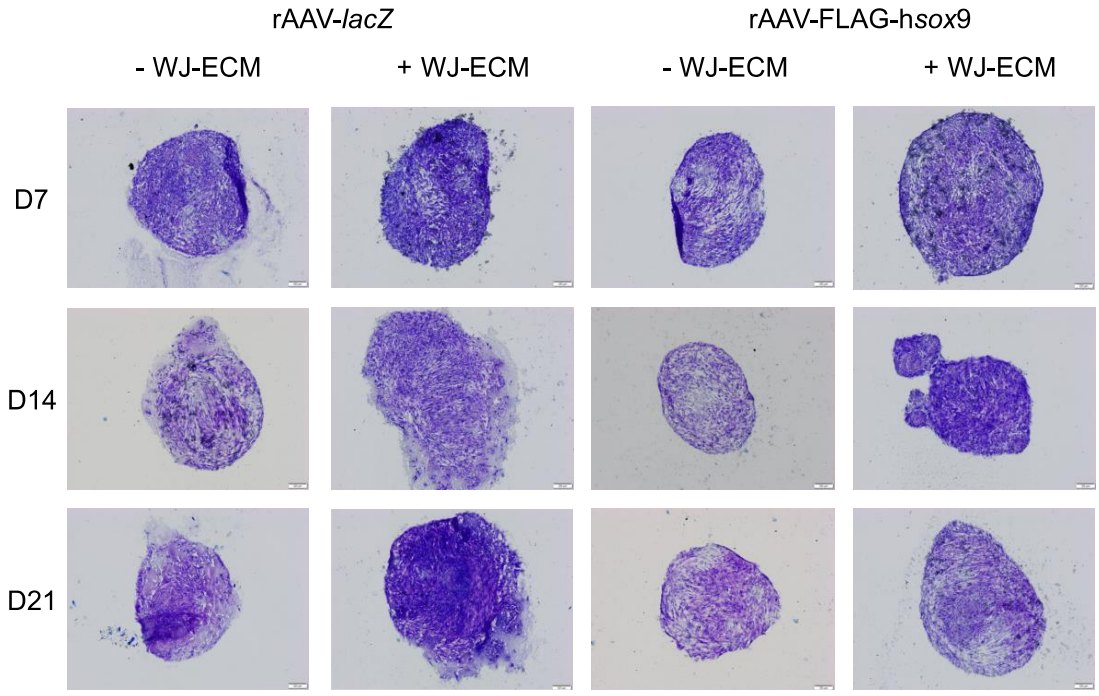

Figure 2. Biological activities in rAAV-transduced chondrogenically-induced hWJ-MSC aggregates in the presence or absence of Wharton's jelly extracellular matrix. Cells in aggregate cultures were transduced with rAAV-*lacZ* or rAAV-FLAG-h*sox9* (40 μl vector, MOI = 4) without or with Wharton's jelly extracellular matrix (-/+WJ-ECM, respectively) and processed after 21 days for toluidine blue staining as described in the Materials and Methods (magnification x100; scale bar = 100 μm; all representative data).

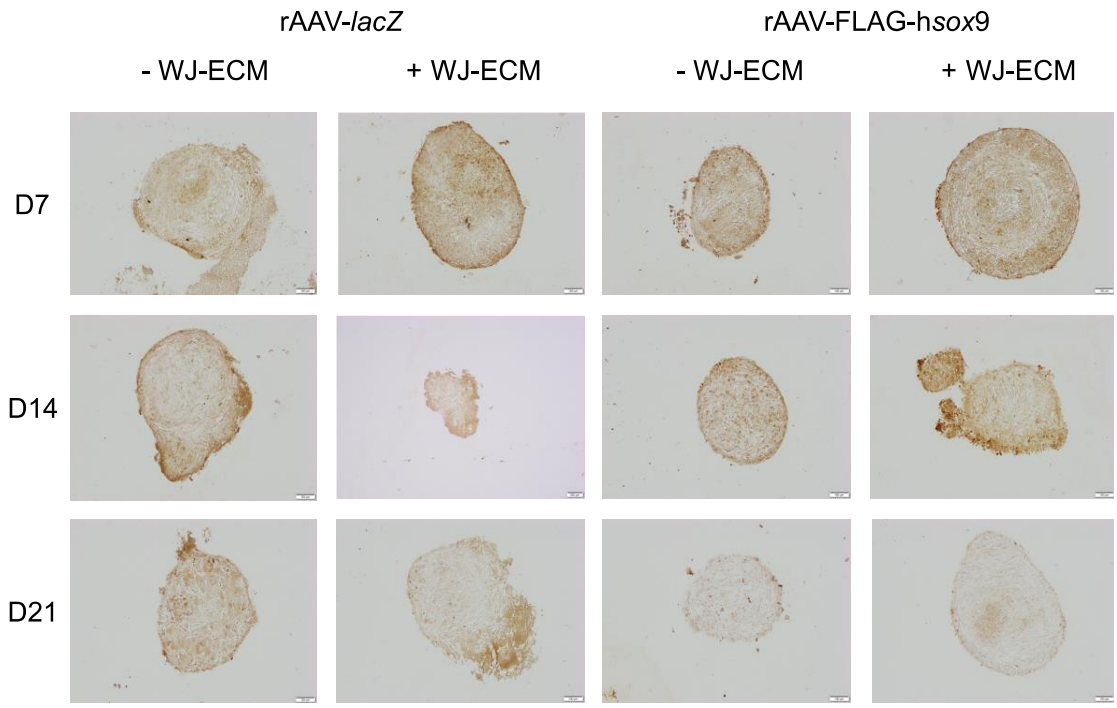

Figure 3. Chondrogenic activities in rAAV-transduced chondrogenically-induced hWJ-MSC aggregates in the presence or absence of Wharton's jelly extracellular matrix. Cells in aggregate cultures were transduced with rAAV-*lacZ* or rAAV-FLAG-h*sox9* (40 μl vector, MOI = 4) without or with Wharton's jelly extracellular matrix (-/+WJ-ECM, respectively) and processed after 21 days to detect type-II collagen expression by immunohistochemistry as described in the Materials and Methods (magnification x100; scale bar = 100 μm; all representative data).

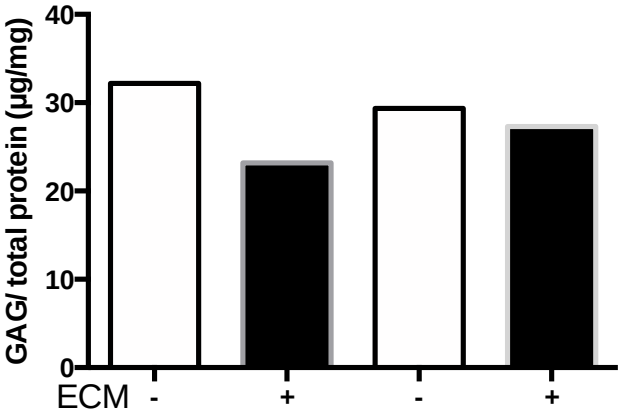

Figure 4. Biochemical analyses after 21 days in vitro. Proteoglycan content standardized to the total protein content of the WJ-MSC aggregates cultured in the presence or absence of WJ-ECM (+/- ECM, respectively).

Modulation of the gene expression profiles in human Wharton's jelly treated with the candidate rAAV-FLAG-hs*ox9* construct using Wharton's jelly extracellular matrix

Chondrogenically-induced aggregate cultures of hWJ-MSCs were finally transduced with rAAV-FLAG-hs*ox9* in the presence of WJECM to investigate the impact of *sox9* gene transfer and overexpression on the levels of cartilage-specific gene expression in the cells in such a natural environment compared with control (rAAV-*lacZ*) treatment, with hBM-MSCs modified and kept in the same experimental conditions, and with conditions in the absence of extracellular matrix.

Application of the rAAV *sox9* vector to the hWJ-MSC aggregates in the presence of WJECM in the cultures increased the levels of SOX9 and ACAN after 21 days relative to rAAV-*lacZ* gene transfer without WJ-ECM (30.7- and 14.3-fold difference, respectively). ACAN level was also increased with rAAV-LacZ gene transfer in the presence of WJ-ECM relative to rAAV-LacZ gene transfer in the absence of WJ-ECM (1à.2-fold difference). No type II- or type X-collagen expression was detected in all tested conditions **(Figure 5)**.

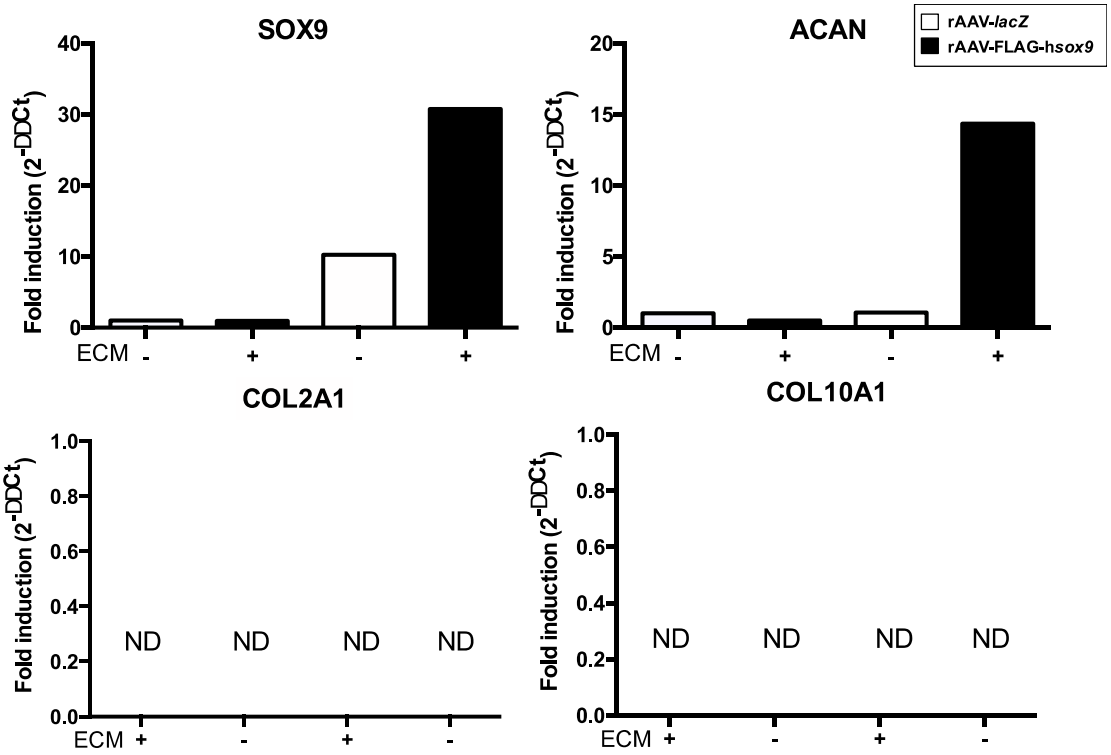

Figure 5. Real-time RT-PCR analysis after 21 days. Samples were processed to monitor the expression of transcription factor SOX9, aggrecan (ACAN), type-II, and -X collagen (COL2A1, COL10A1) in hWJ-MSC aggregates cultured in the presence or absence of WJ-ECM (+/- ECM). Ct values were obtained for each target gene using GAPDH as a control for normalization. Fold inductions (relative to values obtained from the aggregates cultured in the presence of recombinant TGF-β1) were measured by using the 2-ΔΔCt method.

Discussion

Genetic modification of chondrogenically competent progenitor cells, especially those derived from the Wharton's jelly that are more primitive than BM-MSCs [Chen 2014 e98565; El Omar 2014 523], is an attractive approach that may lead to improved setups to enhance cartilage repair by implantation in sites of tissue lesions [Evans 2000 s214; Yoo 2000 s164; Cucchiarini & Madry 2005 1495; Cucchiarini & Madry 2010 135; Frisch 2015 121]. Among many candidate gene sequences (COMP, TGF-β, BMPs, FGF-2, IGF-I, ZNF145, Ihh, siRNA, and miRNA) [Pagnotto 2007 804; Cucchiarini 2011 1921; Liu 2011 2711; Haleem-Smith 2012 1245; Jeon 2012 4413; Steinert 2012 R168; Frisch 2014a 103; Frisch 2014b 1050; Zhang 2017 136 PMID 28123640; Hossein PMID 29040159], we focused on delivering the highly potent SOX9 transcription factor that is well defined for its chondrogenic potency [Bi 1999 85] in hWJ-MSCs via clinically adapted rAAV vectors [Cucchiarini & Madry 2005 1495; Evans & Huard 2015 234; Cucchiarini 2016 495], in their original ECM to mimic a natural, biocompatible cell environment [Dan 2017 227; Dan 2017 s95].

The data first show that rAAV vectors are capable of efficiently and durably promote transgene (*lacZ*) expression in hWJ-MSCs for at least 21 days as observed in hBM-MSCs [Wang 2014 279 PMID 24652327], in good agreement with previous findings using this vector class [Pagnotto 2007 804; Cucchiarini 2011 1921; Venkatesan 2012 22], and probably due to the persistence of rAAV in their targets [Xiao 1996 8098]. Moreover, *lacZ* expression was increased in hWJ-MSCs when maintained in their WJ-ECM. The data also demonstrate that application of rAAV-FLAG-hs*ox9* was able to durably activate the chondrogenic processes in hWJ-MSCs

(proteoglycan and type-II collagen) relative to rAAV-*lacZ*, all in good agreement with the properties of SOX9 [Lefebvre & de Crombrugghe 1998 529; Tsuchiya 2003 338; Goldring 2006 33; Cao 2011 3910; Leung 2011 e1002356; Wang 2014 279 PMID 24652327] and as observed in hBM-MSCs [Venkatesan 2012 22]. The chondrogenic effect was increased in WJ-ECM-treated cells.

In conclusion, the current findings demonstrate the possibility of overexpressing *sox9* via rAAV in hWJ-MSCs as a means to favorably stimulate the chondroregenerative activities of these cells in their native matrix. Such work represents a first, successful step towards translation in clinically relevant (orthotopic) models of cartilage defects [Pagnotto 2007 804; Cao 2011 3910; Liu 2011 2711; Cucchiarini 2013 625; Liu 2017 PMID 28261617] based on the administration of genetically modified WJ-MSCs as a means to enhance cartilage repair *in vivo* and possibly contain hypertrophic events. Such a strategy may have additional therapeutic benefits relative to direct, vector-based therapeutic gene transfer in such a setup [Cucchiarini 2013 625]. Altogether, this study shows the potential of targeting hWJ-MSCs by rAAV gene transfer to overexpress *sox9* in future strategies to treat cartilage lesions in patients.

Conflicts of interest

The authors declare no conflict of interest associated with this work.

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Discussion, Conclusions et Perspectives

Les maladies du système musculo-squelettique, notamment celles touchant les os et le cartilage, affectent plus de 100 millions d'européens. Que ce soit dans le cas de lésions chondrales ou osseuses, les traitements disponibles, qu'ils soient médicamenteux ou chirurgicaux, ne parviennent pas à restaurer les fonctions perdues de manière pleinement satisfaisante. Il est donc nécessaire de développer des stratégies thérapeutiques innovantes telles que celles faisant intervenir la médecine régénérative pour augmenter la régénération osseuse et cartilagineuse chez les patients, car les options cliniques existantes ne peuvent pas restituer pleinement la continuité qui existe entre l'os et le cartilage.

1. La différenciation vasculaire

Les travaux de thèse présentés ici en première partie concernent la différenciation vasculaire de CSM-GW. Si un des objectifs de ce travail était d'accomplir une différenciation chondrogénique afin de produire des implants utilisables pour traiter des lésions chondrales, l'objectif à plus long terme est également de parvenir à créer des implants osseux utilisables pour traiter des lésions osseuses. Dans cette optique, nous nous sommes appuyés sur les compétences dans la différenciation vasculaire du groupe « ingénierie vasculaire » pour différencier des CSM-GW en cellules musculaires lisses afin de mieux maîtriser ce type de différenciation, car une des principales difficultés affectant l'ingénierie tissulaire osseuse est le défaut de vascularisation dans les implants réalisés [7]. L'os est un tissu qui nécessite un très grand nombre de vaisseaux non seulement pour fournir les éléments de base nécessaires à la survie des cellules, mais également pour recruter les précurseurs nécessaires à son remodelage. C'est pourquoi les nombreux essais réalisés dans ce domaine tentent de favoriser l'apparition de cette vascularisation, par exemple en employant des facteurs comme le VEGF permettant de stimuler l'angiogenèse dans l'implant réalisé [38]. Le groupe « ingénierie vasculaire » de l'UMR IMoPA travaille sur l'élaboration de substituts vasculaires notamment pour les pontages coronariens et sur la différenciation des CSM-GW vers des phénotypes vasculaires pour constituer ces substituts. Le groupe est déjà parvenu à différencier les CSM-GW vers un phénotype de CE sur des films multicouches de polyélectrolytes, mais n'avait pas encore travaillé sur la différenciation vers un phénotype de CML. La littérature rapporte de nombreux

essais de différenciation de CSM de tissus adultes à l'aide de facteurs solubles comme le TGF-ß1 [39] et l'acide ascorbique[40]. Ainsi nous avons testé ces stimuli sur les CSM-GW en raison de leurs avantages - comme leurs propriétés immunomodulatrices ou leur plus grande accessibilité - vis-à-vis de CSM originaires de tissus adultes. Après 12 jours de culture *in vitro*, les CSM-GW cultivées en présence d'acide ascorbique ou de TGF-ß1 ont exprimé de nombreux marqueurs protéiques caractéristiques des capacités contractiles des CML vasculaires. Nous avons mis en évidence l'expression de marqueurs précoces de différenciation comme l'alpha-actine du muscle lisse ou la protéine SM22, et de marqueurs intermédiaires comme la calponine et la caldesmone. Cependant la myosine du muscle lisse, un marqueur protéique mature qui permet à la cellule de se contracter, n'était pas exprimée par les cellules, indiquant que la différenciation était initiée mais non complète, et que le phénotype contractile pleinement fonctionnelle n'était pas atteint. En effet, les CML dans les vaisseaux sont entourées d'une MEC sur laquelle elles peuvent prendre appui pour se contracter et qui n'est pas présente *in vitro*. De plus, elles ne sont pas situées dans un environnement mécanique adapté, avec des contraintes d'étirement stimulant leur contraction. Il faut également souligner l'absence de cellules endothéliales dans leur environnement, qui communiquent avec elles notamment par la sécrétion de NO pour favoriser la vasodilatation. Ces éléments environnementaux nous ont conduit à penser qu'un environnement tridimensionnel était indispensable à l'obtention d'un tissu achevé remplissant ses fonctions, et ce quel que soit le tissu, bien que nous ayons démontré que les CSM-GW sont capables d'acquérir un phénotype de CML vasculaire contractile avec des protéines qui leur sont spécifiques.

En conclusion de cette première partie, ces travaux ont permis de démontrer la possibilité d'induire une différenciation vers un phénotype musculaire lisse de CSM-GW, un type cellulaire qui présente plus de nombreux avantageux par rapport aux CSM adultes pour une utilisation en ingénierie tissulaire. Cependant, la différenciation incomplète des CSM-GW a mis en évidence la nécessité de développer un modèle plus complet de différenciation qui tient compte de l'environnement des cellules dans leur tissu, notamment pour la poursuite de nos travaux sur la différenciation chondrogénique des CSM-GW.

2. Différenciation chondrogénique de CSM-GW

Les problèmes rencontrés dans la littérature concernant l'ingénierie du cartilage sont essentiellement liés à la perte du phénotype des cellules différenciées, qu'il s'agisse de chondrocytes adultes ou de CSM différenciées [41]. Nombre d'études s'attachent donc à

modifier certains facteurs de culture pour limiter la dédifférenciation. Ainsi, il a été démontré que l'emploi de certains facteurs de croissance améliorait ou maintenait le phénotype chondrocytaire [42][43]. Au cours de mes travaux préliminaires au ZEO, nous avons réalisé que l'utilisation de facteurs solubles dont la demi-vie est très limitée en culture ne permet pas d'avoir une stimulation de différenciation constante sur les cellules, en parallèle de leur coût élevé. Le ZEO est spécialisé dans le traitements des atteintes ostéo-articulaires par thérapie génique, en employant notamment des rAAV porteurs de gènes induisant la production de facteurs par les cellules transduites. L'intérêt de cette méthode est que les cellules produisent alors elles-mêmes les facteurs de différenciation au cœur même du tissu, de façon continue et sur une durée beaucoup plus longue. L'utilisation de rAAV est alors apparue comme une solution alternative à l'emploi de facteurs solubles dans nos modèles d'ingénierie tissulaire.

La littérature rapporte également l'intérêt d'utiliser des substrats protéiques pour améliorer la différenciation, comme des « coatings » à base de collagène de type I[44] ou II [45]. L'inconvénient est que ces recouvrements sont souvent minces et servent uniquement pour une culture en deux dimensions, en plus de n'être constitués que d'une seule protéine, ne permettant pas de reconstituer la complexité d'une MEC complète. C'est pourquoi aujourd'hui certains emploient directement de la MEC produite par les cellules [46]. En parallèle des travaux sur la différenciation vasculaire, nous travaillons également à Nancy sur l'utilisation de MEC-GW comme support de culture en ingénierie vasculaire. Nous avons démontré que la MEC-GW extraite par méthode enzymatique contient de nombreux facteurs de croissance et protéines matricielles capables de former une couche sur des lames de verre ou des artères ombilicales décellularisées. Au cours de ma thèse, j'ai développé une nouvelle méthode d'extraction de la MEC-GW par broyage mécanique, ce qui m'a permis de m'affranchir de l'utilisation d'enzymes et de l'ajout de sérum de veau fœtal à la solution obtenue. La partie soluble de la MEC-GW peut également être utilisée comme additif de culture dans nos modèles d'ingénierie vasculaire. L'avantage de cette technique est qu'elle repose sur l'utilisation de cordons sur lesquels nous travaillons déjà, en plus d'être récupérés sans aucun dommage pour les patients contrairement à d'autres MEC adultes, et qu'il s'agit ici d'une MEC naturelle qui est susceptible d'interagir plus efficacement avec les cellules que des matériaux synthétiques [47].

Nous avons alors développé un modèle incluant les CSM-GW, la MEC-GW et les rAAV comme vecteur pour induire la différenciation cellulaire. Les CSM-GW ont été cultivées en « pellets » permettant ainsi d'obtenir une architecture en trois dimensions, avec des pellets formés en présence de MEC-GW ou non. La culture de cellules en pellet est déjà fréquemment

utilisée en ingénierie tissulaire pour la différenciation chondrocytaire, car elle permet de cultiver plusieurs types cellulaires en parallèle en plus de permettre de former une structure en 3D [48]. Nos premiers essais se sont portés sur la différenciation chondrocytaire des CSM-GW en nous basant sur l'expertise du ZEO dans ce domaine. Les cellules en suspension ont formé spontanément des pellets, puis ont été transduites avec des rAAV-*sox9* induisant l'expression de SOX9, un facteur de transcription central dans la différenciation du cartilage déjà utilisé dans la littérature pour la différenciation de CSM [49][50], ou des rAAV-*lacZ* permettant l'expression de la ß-galactosidase d'*E. Coli* comme contrôle de transduction. Des « pellets » non transduits ont également été réalisés. Dès 7 jours et jusqu'à 21 jours de culture, nous avons mis en évidence la production de ß-galactosidase dans les pellets transduits avec le rAAV-*lacZ*. Nous avons également mis en évidence la production de collagène de type II et de protéoglycanes dans les pellets transduits avec le rAAV-*sox9*, avec une augmentation notamment en présence de MEC-GW *versus* les pellets sans MEC-GW.

En conclusion, ces résultats démontrent la faisabilité de constituer des greffons pour l'ingénierie articulaire à partir de CSM-GW différenciées en employant les rAAV comme vecteur de transduction. L'ajout de MEC-GW stimule la différenciation des cellules et favorise la sécrétion de matrice cartilagineuse, démontrant son intérêt pour régénérer le cartilage, en plus de potentialiser l'effet de la transduction par les rAAV. De très nombreux essais sont rapportés dans la littérature associant des CSM et/ou des biomatériaux à base de polysaccharides ou de matrices naturelles et/ou de facteurs extérieurs (facteurs de croissance et stimuli mécaniques essentiellement) [9]. À notre connaissance, ces travaux sont les premiers à associer tous ces facteurs et à les combiner à des techniques de thérapie génique pour améliorer la différenciation cellulaire. Nos résultats démontrent la faisabilité d'employer un tel modèle avec la production de matrice cartilagineuse, améliorée notamment par la présence de matrice extracellulaire de gelée de Wharton. Cependant, ces travaux doivent encore démontrer leur efficacité *in vivo*, notamment en ce qui concerne la pérennité du phénotype cellulaire mais également en termes de résistance mécanique car les tissus issus de l'ingénierie tissulaire sont souvent moins résistants que le cartilage natif [51]. La synthèse marqueurs hypertrophiques ou osseux (comme les collagènes de type X et I) n'a pas été détectée, mais devrait également être surveillée au-delà de 21 jours pour s'assurer que l'utilisation des rAAV permet d'obtenir un phénotype stable à long terme, car il a été démontré que le phénotype peut varier plusieurs mois après le début de la culture [52].

En perspective de ces travaux, nous envisageons d'étendre le champ d'utilisation de cette technique en employant les rAAV-BMP4 et -BMP7, produisant des facteurs de différenciation ostéoblastiques et déjà existants au ZEO. En effet, si la faisabilité d'implants cartilagineux est confirmée, ceci permettra de tester la réalisation d'implants osseux par les mêmes moyens. Le ZEO réalise également des rAAV induisant la production de VEGF, ceux-ci pouvant être employés pour tenter d'améliorer et pérenniser la différenciation endothéliale des CSM-GW déjà réalisée à Nancy. De plus, son utilisation conjointe aux rAAV BMP-4 et -BMP-7 pourrait permettre de concevoir des greffons osseux vascularisés en favorisant la formation de vaisseaux dans le néotissu. En effet, il a été démontré que l'utilisation conjointe de la transduction par le VEGF et une autre BMP, la BMP-2, a permis d'augmenter la différenciation ostéogénique [53]. Une utilisation conjointe des rAAV-*sox9* et des rAAV-*vegf* pourrait également permettre de créer un modèle de tissu cartilagineux vascularisé. En effet, dans les cas d'arthrose, un envahissement du cartilage par les vaisseaux de l'os sous-chondral est observé [54]. Il pourrait donc s'avérer intéressant d'avoir un modèle représentatif de cet état pathologique.

Le ZEO travaille notamment avec des CSM de moelle osseuse extraite de patients arthrosiques. De plus, le groupe « ingénierie vasculaire » a reçu l'autorisation de travailler à partir de tissu adipeux issu d'interventions de chirurgie bariatrique, nous permettant ainsi de récupérer les CSM du tissu adipeux. Il serait intéressant de comparer dans notre modèle la différenciation des CSM-GW aux CSM de tissu adipeux ou de moelle issues de patients arthrosiques, toutes deux déjà utilisées dans la littérature [55][56] afin de trouver les cellules les plus adaptées à la régénération tissulaire. De plus, si la différenciation de CSM de moelle de patients malades s'avérait efficace, il serait envisageable d'utiliser les cellules du patient pour réaliser un greffon autologue en s'affranchissant ainsi des problèmes de compatibilité liés à l'implantation de cellules allogéniques.

Nous envisageons également de construire des rAAV permettant l'expression de TGF-ß1, ceci afin de favoriser l'amélioration de la différenciation incomplète des CSM-GW en CML présentée dans l'article n°3 (Partie 2). La culture en trois dimensions de CSM en cours de différenciation en CML pourrait également favoriser l'acquisition d'un phénotype mature.

En ce qui concerne les matrices naturelles, nous travaillons également sur la récupération de la matrice du tissu adipeux (MEC-TA), laquelle pourrait être comparée à la MEC-GW afin de choisir le biomatériau le plus adapté au tissu à régénérer, que ce soit du tissu osseux ou cartilagineux. En effet, il a déjà été rapporté que ce type de matrice pouvait également convenir à l'ingénierie tissulaire du cartilage [57]. Qu'il s'agisse de MEC-GW ou de MEC-TA, la caractérisation de chaque lot de MEC pourrait être effectuée en routine dans une démarche

d’ingénierie tissulaire : soit physiquement par microscopie à force atomique, en permettant d’analyser si les matrices sont capables de constituer un dépôt homogène sur une lame de verre, soit biologiquement grâce au « Proteome Profiler™ Human Antibody Array » qui permettrait d’analyser le contenu en facteurs de croissance, protéines matricielles et protéases de chaque lot de MEC.

A plus long terme, si les résultats se montrent aussi prometteurs dans des essais de différenciation osseuse et vasculaire, nous envisageons de d’optimiser les types cellulaires (GW, tissu adipeux ou moelle) et de matrices (GW, tissu adipeux) les plus adaptés pour chaque type de différenciation. Ils pourraient ainsi être utilisés dans des modèles de coculture [48][41] ou de co-transduction [53] pour constituer des greffons ostéo-cartilagineux à l’aide de rAAV (vecteurs thérapeutiques rAAV-*sox9*, rAAV-*bmp-4*, rAAV-*bmp-7* ou rAAV-*vegf*). Ces implants pourraient être insérés dans des modèles *in vivo* de lésions ostéochondrales créées expérimentalement dans des genoux de lapin. La qualité de la réparation pourrait alors être suivie par analyses histologiques, immunohistochimiques, biochimiques et par RT-qPCR pour évaluer l’efficacité des conditions testées. Les résultats de ces expériences permettraient d'identifier le traitement optimal et les conditions favorables à une réparation osseuse et cartilagineuse. Ceci devrait permettre le développement d'un substitut musculo-squelettique vascularisé à faible coût et facile à obtenir, basé sur le transfert de gène (rAAVs), les CSMs et les matrices extracellulaires naturelles.

Annexes

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MINI REVIEW
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How Do Mesenchymal Stem Cells Influence or Are Influenced by Microenvironment through Extracellular Vesicles Communication?

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Mesenchymal stem cells (MSCs) are widely used in cell therapy and tissue engineering thanks to their self-renewal, their multipotency, and their immunomodulatory properties that make them an attractive tool for regenerative medicine. A large part of MSCs positive effects is due to their secretion products which participate in creating a favorable microenvironment and closely relate these cells to other cell types. Extracellular vesicles (EVs) belong to cellular secretions. They are produced by cells continuously or after stimulation (e.g., calcium flux, cellular stress) and act in tissue homeostasis and intercellular communication. The understanding of the role of EVs is growing, more particularly their impact on cell migration, differentiation, or immunomodulation. EVs derived from MSCs show these interesting properties that may be considered in therapeutics, although they can have adverse effects by facilitating cancer propagation. Moreover, MSC behavior may also be influenced (proliferation, differentiation) by EVs derived from other donor cells. The aim of this mini review is to summarize the two-way communication between MSCs and other cell types, and how they can affect each other with their microenvironment through EVs. On the one hand, the manuscript presents the influence of MSC-derived EVs on diverse recipient cells and on the other hand, the effects of EVs derived from various donor cells on MSCs. The discrepancies between cancer cells and MSCs communication according to the sources of MSCs but also the tumor origins are also mentioned.

Keywords: mesenchymal stem cells (MSCs), extracellular vesicles (EVs), recipient/donor cells, intercellular communication, microenvironment

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INTRODUCTION

At physiological level, the cells composing a tissue, an organ, or even an entire organism are constantly trading information either by physical contact or by long distance communication. This phenomenon allows their maintenance but can also lead to variations in the cellular behavior. The study of these interactions has permitted to develop new therapeutic strategies such as cell therapies.

Thanks to their self-renewal, their multipotency, and their immunomodulatory properties, mesenchymal stem cells (MSCs) are an attractive tool for regenerative medicine. For example, MSCs are used in clinical trials to treat pathologies such as graft vs. host disease (GvHD) (Le Blanc et al., 2008; Zhou et al., 2010; Introna et al., 2014), although concerns remain regarding efficacy, safety, and feasibility of such treatments (Si et al., 2011; Mendicino et al., 2014). The most common sources of MSCs are from adult origin like bone marrow or adipose tissue, but their collects request an invasive procedure. Perinatal sources like Wharton's jelly from umbilical cord offer more accessibility and larger quantities of MSCs with higher proliferation rate and greater immunomodulatory properties (El Omar et al., 2014).

Numerous studies have shown the regenerative potential of MSCs to counteract organ failures (Xing et al., 2014; Desando et al., 2016; Sattayaprasert et al., 2016). It has also been demonstrated that injecting MSC conditioned medium and not the cells themselves can induce the same effects. This is due to the composition of MSC secretions, which are of two types, soluble factors [e.g., soluble tumor necrosis factor receptor 1, transforming growth factor (TGF-β) 1; (Melief et al., 2013; Ke et al., 2016)] or extracellular vesicles (EVs). EVs are involved in tissue reparation, immunomodulation, and proliferation. A miscellaneous EV population can be found in biological fluids. Three kinds of EVs are mostly described by the scientific community according to their size and biogenesis (Höög and Lötvall, 2015; Yáñez-Mó et al., 2015; Kowal et al., 2016). The biggest vesicles are secreted after cell apoptosis and are large EVs from 1 to 5 μm called apoptotic bodies (Atkin-Smith et al., 2015). EVs from 0.1 to 1 μm are termed microparticles, ectosomes, or microvesicles. They are generally produced by cells during stress or metabolic changes and result from the budding of the plasma membrane (Ratajczak et al., 2006). Endosome-derived EVs named exosomes are small EVs—with a size varying from 30 to 150 nm depending on the literature—which are secreted continuously whatever cellular state (stress or physiological conditions) (Valadi et al., 2007; György et al., 2011; Vlassov et al., 2012; Crescitelli et al., 2013). The size similarity between EVs can impair their identification if size is used as the only parameter for characterization. Lately, the difficulties to purify a homogeneous EV population and to select appropriate EV markers to classify them have been discussed and remain. All vesicle populations will be referred as EVs in this mini review and the attention will be highlighted on their biological effects.

MSCs are commonly known as donor cells by providing EVs to other cells types, nevertheless their part as recipient cells is less described (**Figure 1**). A recent report shows that human bone marrow (BM)-MSCs can be both donor and recipient cells. Osteogenically induced BM-MSCs are donors of EVs which are able to guide osteogenic differentiation. The stimulation

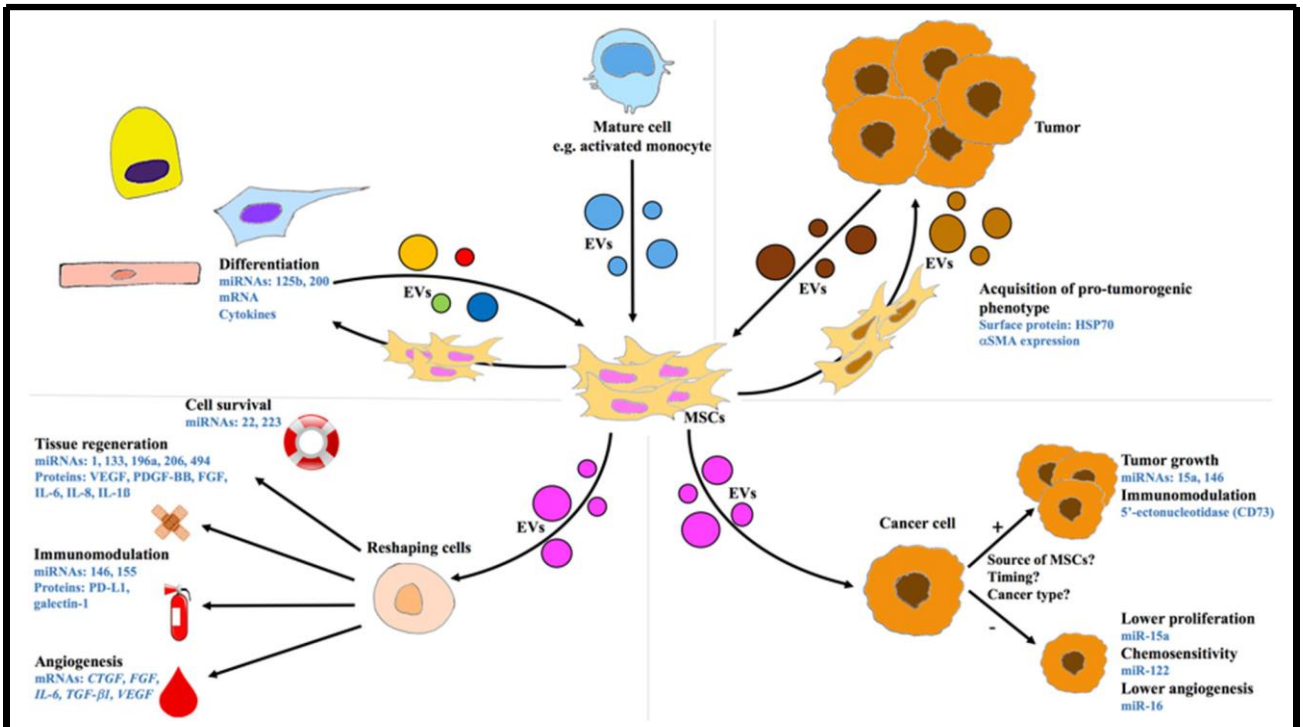

FIGURE 1 | Intercellular communication between MSCs and other cell types through EVs. Upper panels: MSCs as recipient cells. **Lower panels:** MSCs as donor cells. **Left Panels:** MSCs exchanging with differentiated cells. **Right Panels:** Cross-talk between MSCs and cancer cells. CTGF, connective tissue growth factor; EV, extracellular vesicle; FGF, fibroblast growth factor; HSP, heat shock protein; IL, interleukin; mRNA, messenger RNA; miRNA or miR, microRNA; MSC, mesenchymal stem cell; PDGF, platelet-derived growth factor; PD-L1, programmed death-ligand 1; SMA, smooth muscle actin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

of recipient BM-MSCs by these EVs induces expression of bone morphogenic protein (*BMP*)-*2*, *osterix*, osteopontin (*OPN*), osteocalcin (*OCN*), and alkaline phosphatase (*ALP*). Thus, the recipient BM-MSCs may be employed for therapeutic use to improve bone regeneration (Martins et al., 2016). Each cell produces its own secretions which allow the formation of its own microenvironment affecting surrounding cells including MSCs. In this mini review, we focus on the effects of EV exchange between MSCs and other cell types in both ways.

MSCs AS DONOR CELLS

MSC-EVs and Tissue Regeneration

MSC microenvironment acts differently depending on the target cells (**Figure 1**). MSC-EVs can produce modifications in gene expression and mediate cell maturation or differentiation. For example, osteoblasts undergoing stimulation with EVs originated from BM-MSCs show clear calcium deposits *in vitro* in the same way as osteogenic medium stimulation. This is supported by *ALP*, *OCN, OPN,* and runt-related transcription factor (*RUNX*)*2* gene overexpression. These *in vitro* results were confirmed *in vivo* by using hydrogels supplemented with EVs to improve bone regeneration through the expression of micro RNA miR-196a.

MSC-EVs induce angiogenesis stimulation of human umbilical vein endothelial cells by enhancing tube formation in Matrigel^{TM}-coated wells. This observation has been made with fresh or frozen EVs, showing their preservation potential under −80°C (Teng et al., 2015). Montemurro et al. showed that EVs derived from cord blood MSCs carry transcripts related to angiogenic and proliferative function such as connective tissue growth factor (*CTGF*), fibroblast growth factor (*FGF*), interleukin (*IL*)*-6* but also *TGF-ß1,* and vascular endothelial growth factor (*VEGF*) (Montemurro et al., 2016). Transcripts were not the only ones to participate in the pro-angiogenic properties of MSC-EVs. Pro-angiogenic properties beneficial for myocardial tissue repair were highlighted after MSC pre-conditioning by serum starvation plus low oxygen settings in order to mimic ischemic conditions after myocardial infarction. Modification of culture conditions leads to modulation of protein targeting toward MSC-EVs. Specific protein content during stimulation has an effect in angiogenesis and especially by nuclear factor (NF)-κB signaling pathway (Anderson et al., 2016). Intramyocardial injection of MSCs or MSC-EVs also reduces myocardial ischemia in rats 48 h after infarction. Compared to the control, MSCs or MSC-EVs showed similar effects with reduction of the infarct size 28 days after post-operation. Injection of EVs derived from MSCs also improved cardiac function and promoted the number of blood vessels in myocardium infarcted region with a higher increase than MSC injection (Bian et al., 2014). In skeletal muscle, MSC-EVs are able to improve tissue repair. The specific content in growth factors (VEGF, fibroblast growth factor, platelet-derived growth factor-BB), cytokines (IL-1β, IL-6, IL-8), and especially in non-coding RNA may act to maintain a healthy and functional tissue. Three important myogenic micro RNAs (miRNAs: miR-1, -133, and -206) that have been involved in muscle regeneration after injection in rat model appear inside MSC-EVs. Although miR-494 is not specific for muscle repair, it was also found in high concentration inside MSC-EVs. This miRNA participates in myogenesis, migration activity, and protects against ischemia/reperfusion injury in cardiac muscle (Nakamura et al., 2015; Hofer and Tuan, 2016). The potential of MSC-EVs to treat cardiovascular injuries is widely studied. During ischemia, tissue oxygenation becomes almost null, but during this time, MSCs secrete large amounts of EVs containing a high concentration of miR-22 compared to normoxic conditions. Interactions between EVs containing miR-22 and cardiomyocytes allows a reduction of infarcted zone volume and apoptosis in the ischemic myocardium by the down regulation of methyl CpG binding protein 2 (Mecp2) (Feng et al., 2014). Another study highlights the beneficial effect of MSC-EVs on cardiomyocytes survival, this effect being higher when MSCs overexpress transcription factor GATA-4 (Yu et al., 2015). Wang et al. have pointed miR-223 in MSC-EVs as a primordial effector in cardioprotective properties. During polymicrobial sepsis, miR-223 contained inside EVs contributes to the regulation of two proteins, semaphorin-3A (Sema3A) and signal transducer and activator of transcription 3 (Stat3), which are involved in apoptosis. The absence of miR-223 inside MSC-EVs has an impact on cardiac cells and may have deleterious effects. On the contrary, his presence will allow a better defense against sepsis (Wang et al., 2015). Furthermore, MSC-EV regenerative potential has been demonstrated in neurogenesis (Xin et al., 2013), liver fibrosis (Li et al., 2013), and cutaneous wound healing (Zhang et al., 2015).

MSC-EVs and Immunomodulation

MSC-EVs take place in immunomodulation to lower the immune system activation through the induction of anti-inflammatory cytokines and regulatory T cells (Treg) (**Figure 1**) (Del Fattore et al., 2015), but also by regulating macrophages polarization (Ti et al., 2015) and neutrophils mobilization (Zhu et al., 2014). More generally, MSC-EVs have been shown to balance expansion of myeloid progenitors (Goloviznina et al., 2016). MSC-EVs can activate monocytes by Toll-like receptor (TLR) signaling pathway. In contrast with activation by lipopolysaccharides (LPS), the surface receptor involved is unknown but will cause the same signaling cascade via myeloid differentiation primary response gene 88 (MYD88) and NF-κB (Zhang et al., 2014). Another notable difference is observed in cytokine production. Thus, when monocytes are stimulated by MSC-EVs, they differentiate into macrophages which secrete IL-10, leading to Treg expansion. During this study, test of skin allograft rejection by mice treated with MSC-EVs showed similar results with another experiment using cyclosporine A, an immunosuppressor (Zhang et al., 2014). MSC-EVs can induce the decrease of B lymphocyte and natural killer (NK) cell proliferation. Unlike MSCs, EV immunomodulation is not mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO) pathway but by a molecule on their surface: programmed death-ligand (PD-L)1 (Di Trapani et al., 2016). To be activated, MSCs need to be stimulated by pro-inflammatory cytokines to trigger their immunosuppressive answer. Generally, an increase of IDO activity is a marker of activation when MSCs and T cells

are in contact, but during contact between MSC-EVs and T cells, IDO concentration remains stable without modifying the immunological potential. In addition to PD-L1, an endogenous leptin found on EV surface—galectin-1—is also involved in the immunomodulatory response (Del Fattore et al., 2015). Effects of 5′-ectonucleotidase (CD73) has been studied as well because this enzyme found on MSCs and MSC-EVs actively produces adenosine, a molecule known to be immunosuppressive (Kerkelä et al., 2016). Moreover, inflammatory priming induces the increase of miR-155 and -146 level inside MSC-EVs. These specific miRNAs intervene in the regulation of inflammatory reactions (Di Trapani et al., 2016). Clinical applications are also possible to reduce inflammation in some pathologies such as therapy refractory GvHD. Shortly after MSC-EV therapy, cutaneous, and mucosal GvHD showed a very promising response, allowing to reduce four times the administrated steroid doses. Such EV-based treatment have beneficial effects for the patient without side effects (Kordelas et al., 2014).

MSC-EVs in Cross-Talk with Cancer Cells

Despite the numerous studies about pro-angiogenic effects of MSC-EVs, these vesicles may have a reverse effect on cancer cells. A study on mouse breast cancer cell line (4T1) headed by Lee et al. showed that miR-16 level in EVs derived from mouse BM-MSCs contributes to decrease the secretion of VEGF by cells. These modifications lead to a suppression of angiogenesis *in vitro* and thus a reduction in tumor spread (Lee et al., 2013). In the case of cancers therapy with MSC-EVs, there are a lot of divergences between studies because of the source of MSCs, the tumor origin, but also the timing of stimulation with EVs (**Figure 1**). Depending on the papers, MSC-EVs can promote tumor progression (Roccaro et al., 2013; Shi et al., 2016), decrease it (Bruno et al., 2013), or have no effect (Hendijani et al., 2015). In all cases, the content in cytokines and miRNAs seems to be the key factor. The effect of nucleic acids contained in EVs in the cross-talk between tumor cells and MSCs has been well-described by Lopatina et al. (2016). They came to the conclusion that these cells exchange either oncogenic and anti-tumoral RNAs. For example, EVs secreted by multiple myeloma BM-MSCs display a lower tumor suppressive miR-15a content (Roccaro et al., 2013), associated with more cytokines regulating cell adhesion and migration as well as oncogenic proteins. When stimulated with MSC-EVs, cells from nasopharyngeal carcinoma (CNE2) undergo toward a mesenchymal transition, with a decrease of epithelial markers like epithelial cadherin (Shi et al., 2016). MSC-EVs used on different cancer cell lines can also stimulate metalloproteinase (MMP)-2 expression, thus helping tumor migration (Yang et al., 2015). These vesicles have also the ability to transfer CD73 on tumor cells, giving them the ability to metabolize AMP into adenosine, which reduces NK and T cell activation. MSC-EVs can also confer drug resistance to gastric cancer cells by stimulating multidrug resistance protein expression and reducing chemo-induced apoptosis (Ji et al., 2015). However, liver cancer cell lines like HepG2 stimulated with MSC-EVs have difficulties in the cell cycle progression and are subject to apoptosis (Bruno et al., 2013). *In vivo* growth of glioma xenografts is also reduced by miR-146 which is present in MSC-EVs (Katakowski et al., 2013). The chemosensitivity of hepatocellular carcinoma cells was also raised by miR-122 contained in adipose-tissue MSC-EVs. Some cell lines remain unaffected by MSC-EVs, neither in a pro- nor an anti-tumor way. For example, when the lung cancer cell line A549 is exposed to umbilical cord MSCs conditioned medium, it does not lose or gain proliferation rate, even if MSCs are stimulated with interferon γ. The association of conditioned medium with the therapeutic agent doxorubicin does not modify its native effect (Lou et al., 2015). Even if the direct use of MSC-EVs in cancer therapies is still not fully understood and should be carefully controlled, it appears that they could be an interesting vector to address therapeutic cargos to tumors (Chen et al., 2011; Johnsen et al., 2014; Yang et al., 2015).

MSCs AS RECIPIENT CELLS

EVs from Differentiated Cells

EVs derived from differentiated cells are able to modulate MSC phenotype (**Figure 1**). An *in vitro* study has highlighted the potential of EVs derived from neuronal cells to mediate MSC neuronal induction. Indeed, miR-125b—which is known to act in neuronal differentiation—is expressed by MSCs after 1 week of stimulation by these EVs (Takeda and Xu, 2015). When derived from endothelial cells, EVs can influence MSC proliferation, migration, and secretion of soluble factors such as matrix MMP-1, MMP-3, chemokine ligand 2 (CCL-2), and IL-6 (Lozito and Tuan, 2014). In the case of renal tubular morphogenesis and kidney structure, cells known as mesenchymal-epithelial cells are required. The origin of these cells can be explained by the migration and transition of MSCs from bone marrow induced by EVs derived from human renal proximal tubular epithelial cells. The presence of the miR-200 family (miR-200a, -200b, and -200c) has been highlighted in these EVs. Their uptake by BM-MSCs induces a MSC phenotype modification with a mesenchymal to epithelial transition, characterized by the acquisition of polarized epithelial cell properties by BM-MSCs. This is a physiological process involved in kidney formation (Chiabotto et al., 2016). Immune cells such as monocytes communicate also with MSCs via EVs. LPS activated monocytes secrete a lot of soluble factors as well as EVs. The conditioned medium of these cells has the property to modulate MSC phenotype by upregulating osteogenic gene expression (Omar et al., 2011). *RUNX2*, *BMP-2*, and *OCN* expression were evaluated after 72 h of MSC stimulation by conditioned medium or EVs derived from activated monocytes compared to control. During EVs stimulation, *RUNX2* and *BMP-2* were significantly increased compared to control in the same way as conditioned medium but *OCN* was only over expressed with EVs. This indicates that EVs derived from activated monocyte promote osteogenic differentiation in MSCs (Ekström et al., 2013). In a rat model of calvarial bone defect, it has been shown that EVs obtained from induced pluripotent stem cell-derived MSCs associated to a tricalcium phosphate scaffold are able to stimulate bone regeneration by recruiting BM-MSCs at the defect site. The rat's BM-MSCs are activated through the phosphatidylinositol 3

kinase (PI3K)/Akt pathway leading to osteogenic differentiation (Zhang et al., 2016).

EVs from Cancer Cells

Genetic modifications of MSCs are also possible via EV transfer from cancer cells (**Figure 1**). When EVs are originated from healthy cells, their effects seem beneficial. However, when EVs come from cancer cells, their influence on MSCs may be harmful. Lindoso et al. have demonstrated that EVs derived from renal cancer stem cells can induce epigenetic changes in recipient cells. MSCs are attracted to the tumor region and change their phenotype, becoming pro-tumorigenic. This correlates with the overexpression of genes involved in cell migration: *chemokine receptor type (CXCR)4* and *CXCR7*; matrix remodeling: *collagen type IV alpha 3 chain (COL4A3)*; as well as angiogenesis and tumor growth: *IL-8*, *OPN,* and *myeloperoxidase*. EVs secreted by cancer stem cells allow a better chemoattraction of MSCs, which promote tumor development and spread (Lindoso et al., 2015). There are also differences in the evolution of MSC phenotype which is reliant on the origin of EVs. Thus, MSCs can differentiate into myofibroblasts under action of EVs from prostate cancer. The myofibroblastic marker alpha-smooth muscle actin α-SMA) is expressed by more than 50% of MSCs exposed to prostate cancer derived EVs vs. only 5% of cells under TGF-ß1 stimulation, which is known to induce α-SMA expression. There is also a correlation between the quantity of EVs and α-SMA acquisition (Chowdhury et al., 2015). This differentiation has also been demonstrated with EVs from breast cancer cells or chronic lymphocytic leukemia (Cho et al., 2011; Paggetti et al., 2015). Another example of MSC phenotype modification has been highlighted by Li et al. during a study about lung tumor EVs. *In vitro,* EVs from the lung cancer line A549 stimulate the production and secretion of inflammatory cytokines in MSCs. Three cytokines: IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were released by MSCs when they are triggered by A549 cell-derived EVs. The priming of MSCs by lung cancer cell-derived EVs would occur through the activation of TLR2/NF-κB signaling by the interaction of EVs surface heat shock protein 70 (HSP70) with cells (Li et al., 2016). In a different experiment, EVs from KMBC (human cholangiocarcinoma cells) enhance modifications of MSCs toward a fibroblastic phenotype. Such transformation is not the only change: MSCs brought into contact with KMBC-EVs subsequently secrete IL-6 which causes an increase in cancer cells proliferation (Haga et al., 2015). Chronic myelogenous leukemia (CML) uses the same pathway to spread. CML-EVs interact with BM-MSCs and this stimulation initiates IL-8 production by MSCs which permits CML cells proliferation (Corrado et al., 2014). The cross-talk between MSCs and cancer cells leads to a specific sequence. First, cancer cells interact with MSCs via their EVs to modulate them and in response, the modified MSCs participate in cancer progression trough their own EVs (Corrado et al., 2014; Haga et al., 2015).

CONCLUSION

Cellular communication by EVs relies on the nature of donor cells and recipient cells (**Figure 1**). Research on MSC-EVs is prolific and shows their potential in regenerative medicine, whereas there is less literature about EVs originated from healthy differentiated cells. For these last, the difficulties may originate from a low proliferation and a poor EV production *in vitro*. In the case of cancers, the mechanisms of the cross-talk between the tumor and MSCs are yet not fully unraveled, but some works on EVs as therapeutic cargo show promising results.

AUTHOR CONTRIBUTIONS

GD discussed the work and wrote the manuscript. BM helped to write the manuscript and designed the figure. PM helped to discuss the work and to edit the manuscript. EV drafted the manuscript, discussed the work, edited the manuscript, and gave the final approval of the version to be published.

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Original Research Article

Nanoliposomes of marine lecithin, a new way to deliver TGF-β1

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Abstract

Studies made these last decades showed how TGF-βs family molecules have a crucial role in cell signalling in order to stimulate the differentiation of stem cell into desired phenotype in time and dose dependence, making them essential in regenerative medicine. Therefore, controlling TGF-βs cell administration during culture or healing is essential. Thus, we focused on a nanoliposome as natural nanocarrier for drug delivery system used since few years in pharmaceutics and cosmetics applications. This work studied at first culture effects on human mesenchymal stem cells (hMSCs) harvested from Wharton's Jelly human umbilical cord of TGF-β1 encapsulated or not into salmon lecithin nanoliposomes from 0 to 2.5ng/mL up to 14 days. During culture, cell metabolic activity, proliferation and shape were investigated. In parallel, salmon lecithin and nanoliposomes were analysed according to routine physico-chemical analyses such as lipid composition, nanoliposome size and morphology. These analyses showed that salmon lecithin was composed of a high level of polyunsaturated fatty acids essentially EPA and DHA. Moreover, neither nanoliposome nor TGF-β1 alone or encapsulated into nanoliposome showed any cytotoxicity according to the concentration studied herein. Finally, nanoliposome solutions have a higher impact on cell proliferation than TGF-β1 and compared to TGF-β1 alone, these solutions limit maximum cell number and maintain a higher level cell metabolic activity. In conclusion, this study represents a first experimental approach based on morphological cell and cytocompatibility which showed no *in vitro* negative effect on hMSCs.

Keywords: Nanoliposome, TGF-β1, hWJ-MSC, metabolic activity, cell proliferation, physico-chemical analyses

INTRODUCTION

Transforming growth factor (TGF-β) is a described protein in mammal with three isoforms, TGF-β1, -β2 and -β3. Associate with latent TGF-beta binding protein (LTBP) and latency-associated peptide (LAP), TGF-β will be transported and stored in inactive condition. To be active, the two-polypeptide chains must be removed (Annes, 2003; Jobling et al., 2006). This is possible by the presence of different proteins such as matrix metalloproteinase-2 (MMP-2), MMP-9, bone morphogenetic protein-1 (BMP-1) but also by the environment (pH) and reactive oxygen species (ROS) (Ge and Greenspan, 2006; Yu and Stamenkovic, 2000). TGF-β1 have implication in several processes such as proliferation

control (Miller and Luo, 2002), differentiation (Narita et al., 2008) and metabolic activities (Hosseinzadeh et al., 2014) in most mammal cells. To stimulate metabolic activities of cells, the proteins interact with transmembrane glycoproteins: TβRI and TβRII. A receptor complex containing two of type 1 and type 2 subunits will permit the activation of signalling pathway (Shi and Massagué, 2003). Several pathways are accessible by this mechanism dependent or not from Smad family members, Smad 2/3, Smad 4 and Smad 6/7 (Horbelt et al., 2012). When the Smad route is activated, transcriptional activation can be made with Smad playing a role of co-repressor or co-activator (Massagué, 2000). TGF-β1 action is determined by its concentration, its time exposure, the targeted cells and some potential stress conditions. Thus, it has been shown that this growth factor may be used to stimulate differentiation of stem cells towards a desired phenotype that makes it a perfect element for tissue engineering (Kinner et al., 2002; Zhao and Hantash, 2011).

As TGF-β1 has cell effects in concentration and time dependence, it is essential to control its administration to cell culture. To do so, TGF-β1 could be encapsulated into carrier as drug delivery system. A new method to encapsulate active molecules has recently emerged using lipid nanocarriers: nanoliposomes (Hasan et al., 2016, 2014; Kadri et al., 2016; Latifi et al., 2016; Maherani et al., 2013). Their compositions essentially made of phospholipids that permit self-sealing in aqueous media and make them a good carrier system in biology. These nanoliposomes can be produced using lecithin from soya, rapeseed or salmon. They are biocompatible and their composition in fatty acids play a role in cell metabolism (Arab Tehrany et al., 2012). All these characteristics are already use in food (Reza Mozafari et al., 2008), cosmetics and pharmaceutics (Díaz and Vivas-Mejia, 2013) industries and can be useful for tissue engineering as micro, nano-carrier for active molecules both hydrophilic and hydrophobic to control cell proliferation and cell phenotypes such as for mesenchymal stem cells (MSCs) (Kinner et al., 2002; Zhao and Hantash, 2011).

Indeed, MSCs are more and more used in regenerative medicine for their interesting properties such as good in vitro proliferation and their pluripotency. Thus, under specific stimulations, they can differentiate into several cell types such as chondrocytes (Bertolo et al., 2015), adipocytes (Amable et al., 2014), osteoblasts (Shen et al., 2015), endothelial cells (Ikhapoh et al., 2015) or smooth muscle cells (Kurpinski et al., 2010). They are found in adult tissues bone marrow and peripheral blood but also in embryonic annexes such as placenta and Wharton's jelly (WJ). For many reasons such as immunomodulation, differentiation capacity and availability, WJ-MSCs are becoming the best candidate to develop new therapies (El Omar et al., 2014).

In this study, we have focused on nanoliposomes made of salmon lecithin, TGF-β1 and TGF-β1 nanoliposome encapsulated. Thus, physic-chemical analyses were conducted to characterise these carriers in term of size, structure, stability, etc. After these analyses, different stimulations were added on WJ-MSCs *in vitro* up to 14 days and their biological effects on metabolic activity, cell proliferation and shape were evaluated.

Experimental

Physico-chemical analyses and nanoliposome fabrication process

Salmon lecithin from Salmo salar, was provided by enzymatic hydrolysis. The lipids were extracted by use of an enzymatic process without any organic solvent described by Linder et al. (Linder et al., 2002). BF3 (Boron trifluoride)/methanol, chloroform, hexane, acetonitrile, methanol and other chemicals were obtained from Sigma-Aldrich (France) and Fisher (France). All the organic solvents were analytical grade reagents.

Fatty acids composition

Fatty acid methyl esters (FAMEs) from salmon lecithin were prepared as described by Ackman (Ackman, 1998). Then, the FAMEs were analysed using a Shimadzu 2010 gas chromatography system (Shimadzu, France) equipped with a flame-ionization detector. Separation of FAMEs was accomplished on a fused silica capillary column (60m, 0.25mm i.d. ×0.20µm film thicknesses, SPTM2380 Supelco, Bellfonte-PA-USA). Injector and detector temperatures were settled at 250°C. The column temperature was fixed initially at 120°C for 3 minutes, then raised to 180°C at a rate of 2°C.min^{-1} and maintained at 220°C for 25 min. Individual polyunsaturated fatty acids (PUFAs) were identified using standard mixtures (PUFA1 from a marine source and PUFA2 from a vegetable source; Supelco, Sigma-Aldrich, Bellefonte, PA, USA). The results were showed as triplicate analyses.

Characterisation of lecithin for lipid classes

The lipid classes of different fractions of lipid from salmon lecithin were determined by Iatroscan MK-5

TLC-FID (Iatron Laboratories Inc., Tokyo, Japan). The measurement was performed according to the protocol described in detail in a previous paper (Arab Tehrany et al., 2012). Two migrations were done to determine the proportion of neutral and polar lipid fractions. All standards were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany). Area percentages were presented as the mean value of three repetitions.

Preparation of nanoliposomes solutions

In order to prepare nanoliposome solutions, 20mg of marine lecithin were added into 1980µL of distilled water to obtain a solution with 1% (w/w) marine lecithin. The solution was stirred for 4 hours to obtain a homogenous suspension of lecithin into water and then sonicated for 120 seconds (40Hz, 40% of full power). The solution was then stored at 4°C and protected from light until it was mixed into cell medium. For the nanoliposome TGF-β1 encapsulated solutions respectively at 1250ng/mL, 500ng/mL and 250ng/mL of TGF-β1 (cat. 100-21, PeproTech, USA) and 1% (w/w) of marine lecithin, TGF-β1 solution into distilled water were prepared and added to marine lecithin replacing distilled water of marine lecithin nanoliposome solution protocol presented here above.

Liposome size and electrophoretic mobility measurements

The liposome size was analysed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern instruments, UK). The apparatus was equipped with a 4mW He/Ne laser emitting 633nm, measurement cell, photomultiplier and correlator. The samples were diluted (1:400) with ultra-filtrate distilled water and placed in vertical cylindrical cells (10mm-diameter). The scattering intensity was measured at a scattering angle of 173° relative to the source using an avalanche photodiodes detector at 25°C. Intensity autocorrelation functions were analysed by a General Purpose Algorithm (integrated in the Malvern Zetasizer software) in order to determine the distribution of translational z averaged diffusion coefficient of the particles, DT ($m^2.s^{-1}$). The DT parameter is related to hydrodynamic radius (Rh) of the particles through the Stokes–Einstein relationship $DT = kBT/(6\pi\eta Rh)$. In dispersion, particles are in a constant random Brownian motion, which causes the intensity of scattered light to fluctuate as a function of time. Therefore, droplets sizes were obtained from the correlation function calculated by the dispersion technology software (DTS) using various algorithms. The refractive index (RI) and absorbance were fixed at 1.471 and 0.01 at 25°C, respectively. The measurements were performed in five repetitions.

Stability of nanoliposomes

The nanoliposomes were stored in a drying-cupboard at 37°C for 30 days. Mean particle size, electrophoretic mobility and polydispersity index were analysed every 3 days.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy was employed to monitor the microstructure of the nanoliposome samples with a negative staining method. The samples were diluted 30-folds with distilled water to reduce the concentration of nanoliposomes. Equal volumes of the diluted sample and a 2% ammonium molybdate solution were combined and left for 3 minutes at ambient conditions. A drop of this solution was placed on a Formvar-carbon coated copper grid (200 mesh, 3mm diameter HF 36) for 5 minutes. The excess liquid was drawn off by filter papers. After drying the grid at room temperature for 5 minutes, micrographs were made using a Philips CM20 operating at 200kV and recorded using an Olympus TEM CCD camera (Arab Tehrany et al., 2012).

Membrane fluidity

Fluorescence polarization (P) was used to determine membrane fluidity of liposomes by measuring the fluorescent intensity of TMA–DPH, a compound that contains a cationic trimethylammonium (TMA) substitute that acts as a surface anchor to improve the localization of the fluorescent probe of membrane interiors, diphenylhexatriene (DPH).

This measurement was carried out according to the conventional method (Maherani et al., 2012). The solution of TMA–DPH (in ethanol) was added to the liposome suspension to maintain the lipid/probe molar ratio of 250 ([TMA–DPH] final = 8µM). The mixture was then incubated for at least 1h at ambient conditions with gentle stirring and distributed into 96-well black microplate at 200µL per well. The fluorescence probe was vertically and horizontally oriented in the lipid bilayer. The fluorescence intensity of samples was measured with Tecan INFINITE 200R PRO (Austria) equipped with fluorescence polarizers. Samples were excited at 360nm and emission was registered at 430nm under constant stirring at 25°C. The software used was

Mgellan 7 from Tecan group LTd (Switzerland). The P value of TMA–DPH was calculated from the following equation:

$$P = \frac{I_{//} - GI_{\perp}}{I_{//} + 2GI_{\perp}} \quad (1)$$

where $I_{//}$ is the intensity of fluorescence parallel to the excitation plane, $I_{\perp}$ is the intensity of fluorescence perpendicular to the excitation plane, and G is the factor that accounts for transmission efficiency. The reciprocal value of polarization (1/P) was defined as the membrane fluidity.

Biological Analyses

Cell Mediums

In order to prepare cell medium hereinafter called complete medium, 10% of fetal bovine serum, 2mM of L-glutamine, 2.5g/L of Fungizone® and 100 IU/mL of penicillin and 100 µg/mL streptomycin were added to α-MEM culture medium. Nanoliposome and nanoliposome TGF-β1 encapsulated mediums were prepared by mixing a nanoliposome solution with complete medium in a ratio 1:500 to obtain complete medium with respectively 20µg/mL of marine lecithin and 0, 0.5, 1 or 2.5ng/mL encapsulated TGF-β1. The prepared medium solutions were then filtered with a 0.22µm pore size filter before use.

Isolation and expansion of human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) were collected from Wharton's jelly from connective tissue of human umbilical cord. The latter is considered as a surgical waste and is destroyed outside the context of research. In this case, the umbilical cords were dedicated to the experiments after mother information and agreement applying the guidelines ratified by the University Hospital Center of Nancy (agreement TCG/11/R11). Its use doesn't require any statement ahead the human ethics committee.

Cells were collected after enzymatic digestion of the WJ using collagenase solution at 1mg/mL (Sigma-Aldrich, France) during 18 hours at 37°C. Cells were collected and cultivated in an incubator at 37°C and 5% CO_2 with complete medium. Culture medium was change each 3 days until passage 4 (P4).

Cell culture conditions

At P4, hMSCs were sub-cultivated and seeded into 96-well plates for LDH and MTT assays and 12-well plates for DNA assay at a density of 1 000 cells/cm². Cells were cultured until day 14 with filtered (0.22 µm) culture medium supplemented with TGF-β1 at 0.5, 1 and 2.5ng/mL or with marine lecithin nanoliposomes at 20µg/mL encapsulating 0, 0.5, 1 and 2.5ng/mL of TGF-β1. A group of cells was cultured only with culture medium as control. At day 3, 7 and 14, cells were analysed in triplicate (n=3) for MTT and DNA assays and at day 1 and 7 (n=1) for immunofluorescence imaging of cell nuclei and actin cell filaments.

LDH assay

The LDH assay was realized 24 hours after the stimulations using the Cytotoxicity Detection Kit: LDH (Roche). This assay measures the level of lactate dehydrogenase (LDH) in the medium, which is an intracellular enzyme released at cell death.

As positive control, 5µL of detergent (Triton™ X-100; GIBCO, France) in a seeded control well was added to lysate cells and measure the 100% LDH level. For the other wells, 100µL of supernatant were transferred on a new 96-well plate and 100µL of LDH Kit solution were added, then incubated away from light during 30 minutes under slow stirring. Absorbance of each wells were then measured at the wavelength of 490nm.

DNA Assay

Cell proliferation was evaluated by DNA concentration measurement as already described in Poleni *et al.* (Poleni et al., 2010). In brief, cells of 12-well plates at each time point were washed 3 times with phosphate buffered saline (PBS) and trypsinized with 0.025% of Trypsin-EDTA then centrifuge at 300*g* during 10 minutes. The cell pellets were collected then frozen at -80°C until measurement. All samples were measured in the same time; pellets were suspended into 100µL of Hoechst buffer. Cells were then lysate by 3 cycles of frozen-unfrozen using liquid nitrogen and a water-bath at 37°C. Samples were then completed to 1mL with Hoechst 33258 (Invitrogen, molecular probes, France) solution and absorbance were measured at 456nm. In parallel, a DNA standard curve (not shown here) of absorbance was made using calf thymus DNA (Sigma-Aldrich, France) solutions at different concentrations from 0 to 1µg/mL of DNA with Hoechst buffer and solution.

MTT assay

Cell metabolic activity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

staining (MTT; Sigma-Aldrich, France) prepared in PBS at a concentration of 0.5% (w/v). For each time point, the tested wells had their culture medium removed and were washed with PBS. Then in each well, plus an empty well as negative control, 50µL of MTT solution was added and incubated at 37°C with 5% CO_2 for 4 hours. Then the supernatant was removed and 200µL of dimethylsulfoxide (DMSO; Fisher Scientific, France) were added to each well and incubated for 5 minutes away from light at 37°C. In a new 96-well plate, 100µL of solution were transferred from each tested well and the absorbance was measured at 570nm with a reference wavelength of 700nm.

Fluorescent microscopy imaging

For imaging cells' nucleus and F-actin filament, cells of each well were washed twice with PBS then fixed with 4% of paraformaldehyde solution during 15 min at 37°C. After then, cells were washed 5 min with PBS and permeabilised with 0.5% of Triton X-100 in PBS during 15 min. Cells were then washed twice in PBS during 5 min and incubated during 30 min with phalloidin solution 1:100 in PBS with 0.1% of Triton X-100 and 0.5% of BSA. Then after, cells were washed three times with PBS and incubated during 30 sec with DAPI solution at 1:25000 in PBS. At the end, cells were washed three times with PBS and imaging using Leica DMI3000 D microscope.

Statistics

All data are presented as Mean ± Standard Deviation. Statistical significance was determined by a one-way ANOVA tests followed by a Tukey's post-hoc ($p<0.05$). Student t-test was used when comparing non nanoliposome group to nanoliposome group or TGF-β1 group to non TGF-β1 group ($p<0.05$).

RESULTS AND DISCUSSION

Physico-chemical analyses

Fatty acid

The first observations allow us to highlight that the total polyunsaturated fatty acids in salmon lecithin were predominate with 46.28%. The percentage of the fraction of PUFAs is followed directly by the percentage of saturated fatty acids (SFAs) and of monounsaturated fatty acids (MUFAs, 20.32%) in salmon lecithin (33.40%).

Among various PUFAs, the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is significantly higher compared to other PUFAs with 11.03 and 26.26%, respectively (Table 1).

Lipid classes

The lipid classes of lecithin were separated by thin-layer chromatography (Iatroscan). We identified various phospholipids like phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SPM). At that stage, PC represented the major class of phospholipids contained in salmon lecithin (42.4 ± 0.5 %). Moreover, the percentage of triacylglycerols (TAGs) contained in rapeseed lecithin was 31.2 ± 0.8%.

Liposome size and electrophoretic mobility

Mean size, size distribution and z-potential values are physicochemical parameters that have to be modulated as a function of the proposed application for a certain liposomal system (Grazia Calvagno et al., 2007). The particle sizes of nanoliposomes were measured immediately after preparation. The minimum size that can be achieved depends on the viscosity of the materials and on the applied sonication and homogenization parameters. The average liposome diameter was 100.1 ± 0.6nm for particles from salmon lecithin. The polydispersity index (PI) is a dimensionless measure of the breadth of the particle size distribution (Zweers et al., 2003). When the PI value is <0.3, the indication is that the sample is of narrow distribution, and when PI value is over 0.3, the sample is considered to have a broad distribution (Yen et al., 2008). The polydispersity index was 0.22 ± 0.01 for salmon nanoliposomes. Thus, it was clear that the size of the nanoliposome depended not only on such physical parameters but also on fatty acid composition, lipid classes and the surface-active properties of lecithin (Arab Tehrany et al., 2012; Benedet et al., 2007). Electrophoretic mobility values varied between -3 and -4µm.cm.$V^{-1}s^{-1}$ with a relatively high stability of the formulations, which was due to the positive and negative charge of the polar fraction of lecithin. According to the DLS results, the zeta potential was -45.8 ± 2.4mV for salmon lecithin. It should be noted that the value of zeta potential was negative throughout the storage period for nanoliposome formulation. The salmon lecithin contains different type of phospholipids like PS, phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), PE and PC.

At physiological pH, these phospholipids are negatively charged except the PC and PE which exhibits no net charge. So, these anionic fractions are probably responsible for the negative electrophoretic mobility (Chansiri et al., 1999). We measured the stability of nanoliposomes by measuring the particle size during one

Table 1. Fatty acid compositions of salmon lecithin.

Fatty acids	Salmon lecithin	
	%	SD
C14	2.72	0.04
C15	1.13	0.01
C16	20.67	0.15
C17	0.88	0.05
C18	6.10	0.05
C20	0.35	0.03
C22	1.55	0.06
SFA	**33.40**	
C14:1n9	0.33	0.02
C16:1n7	3.23	0.07
C18:1n9	13.87	0.13
C20:1n9	2.11	0.06
C22:1n9	0.79	0.08
MUFA	**20.32**	
C18:2n6	1.20	0.04
C18:3n3	0.35	0.01
C20:4n6	3.52	0.02
C20:5n3(EPA)	11.03	0.13
C22:5n3	3.93	0.33
C22:6n3(DHA)	26.26	0.09
PUFA	**46.28**	
n-3/n-6	8.88	
DHA/EPA	2.38	

month at 37°C. We observed no significant variation between day 0 and day 30.

Morphology and size of the liposomes

TEM images indicated that vesicles prepared by sonication and high-pressure homogenization method were in the form of multilamellar vesicles (MLV) because of the sonication and homogenization steps. The bilayer nature of the vesicles was clearly visible in these micrographs (Figure 1). We also observed some droplets in each formulation because of the presence of weak oil quantity (10%). The droplets in the three systems were found to be fine enough, even though there were large droplets with diameters higher than 100nm.

Membrane fluidity

The fluidity of lipid membrane also reflects the order and dynamics of phospholipid alkyl chains in the bilayer and liposome permeation (Maherani et al., 2012). The influence of liposome composition on membrane fluidity was investigates by Coderch et al. (Coderch et al., 2000) and Calvagno et al. (Grazia Calvagno et al., 2007). They determined a significant difference in the release profiles of drugs by the presence of two factors: (i) the strength of the drug–liposomal lipid interaction and (ii) the fluidity of the bilayer, i.e. the drug leakage to the aqueous solution increased by increasing the fluidity of the bilayer. The presence of saturated FAs increased the packing between phospholipids, which expelled the water in the vicinity of the bilayer surface, which was evidence of reduced membrane fluidity. On the other hand, unsaturated FAs helped reduce the packing between phospholipids and preserved the level of hydration, thus maintaining membrane fluidity (Leekumjorn et al., 2009). The membrane fluidity of salmon nanoliposome was 324 ± 0.05. We can observe that membrane fluidity depends on lipid composition of nanoliposomes.

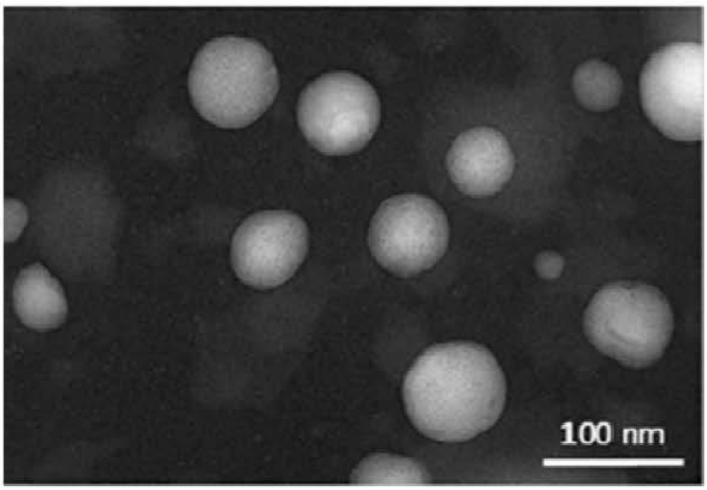

Figure 1. Transmission electron microscopic images of nanoliposomes

Biological analyses

Nanoliposome harmlessness

To examine cell toxicity toward the selected nanoliposome and TGF-β1 concentrations, a LDH release test was made after 24 hours of exposure, whatever stimulation. No statistical significant difference of LDH release was observed between the different conditions and the control (data not shown). Thus, no cytotoxicity of these molecules was found for the concentration used.

Cell proliferation

In order to examine cell proliferation of the different groups, DNA level for each well were measured at day 3, 7 and 14 using Hoechst dye and calf DNA concentrations for standard curve (Figure 2A). For all groups, the concentration of DNA was increased at different rate except for the nanoliposome group (NLP) displaying a slight decrease between day 7 and day 14 which could be related to a stabilisation of cell population. These observations assess a cell proliferation for each group and confirm the results obtained with microscopy imaging and LDH release test of non-toxicity. However, even if there were no significant differences, a tendency of higher proliferation between day 3 and day 7 appeared for NLP and TGF-β1 alone at 0.5ng/mL with a stabilisation of cell number after day 7 compared to control. Moreover, the presence of nanoliposomes regulates significantly the number of cells at day 14 compared to other groups ($p<0.001$). Thus, nanoliposomes have more impact on cell proliferation than TGF-β1, except for the highest concentration, by increasing the rate of cell proliferation at an early stage and by limiting the maximum number of cells compared to control or TGF-β1 without nanoliposome groups.

Cell metabolism

In order to examine cell metabolic activity, MTT test were done at day 3, 7 and 14. The results of MTT test were then normalized by DNA content to decouple metabolic activity and proliferation from the measure. The results presented in figure 2B showed that most groups induce a high decrease of cell metabolic activity between day 3 and day 7. This was followed by the stabilisation of their metabolism until day 14 for cells in contact with nanoliposomes independently of TGF-β1 concentration and a decrease for the other groups except for TGF-β1 0.5ng/mL which metabolic activity level was already low at day 3 and maintained thereafter. Thus, the presence of TGF-β1 into the medium significantly decrease earlier metabolism activity level compared to other groups ($p=0.001$ at day 3). Moreover at day 14, TGF-β1 1ng/mL group had a significantly lower metabolic activity than the one with TGF-β1 1ng/mL nanoliposome encapsulated. Thus, we hypothesis that nanoliposome encapsulation regulates TGF-β1 by progressively deliver it into the medium than without encapsulation which delayed the decrease of cell metabolic activity. Overall, cells which were in contact with nanoliposomes had, at day 14, a higher metabolic activity level than the others ($p=0.0016$).

Fluorescent microscopy imaging

The microscopy imaging made at day 1 and day 7 by

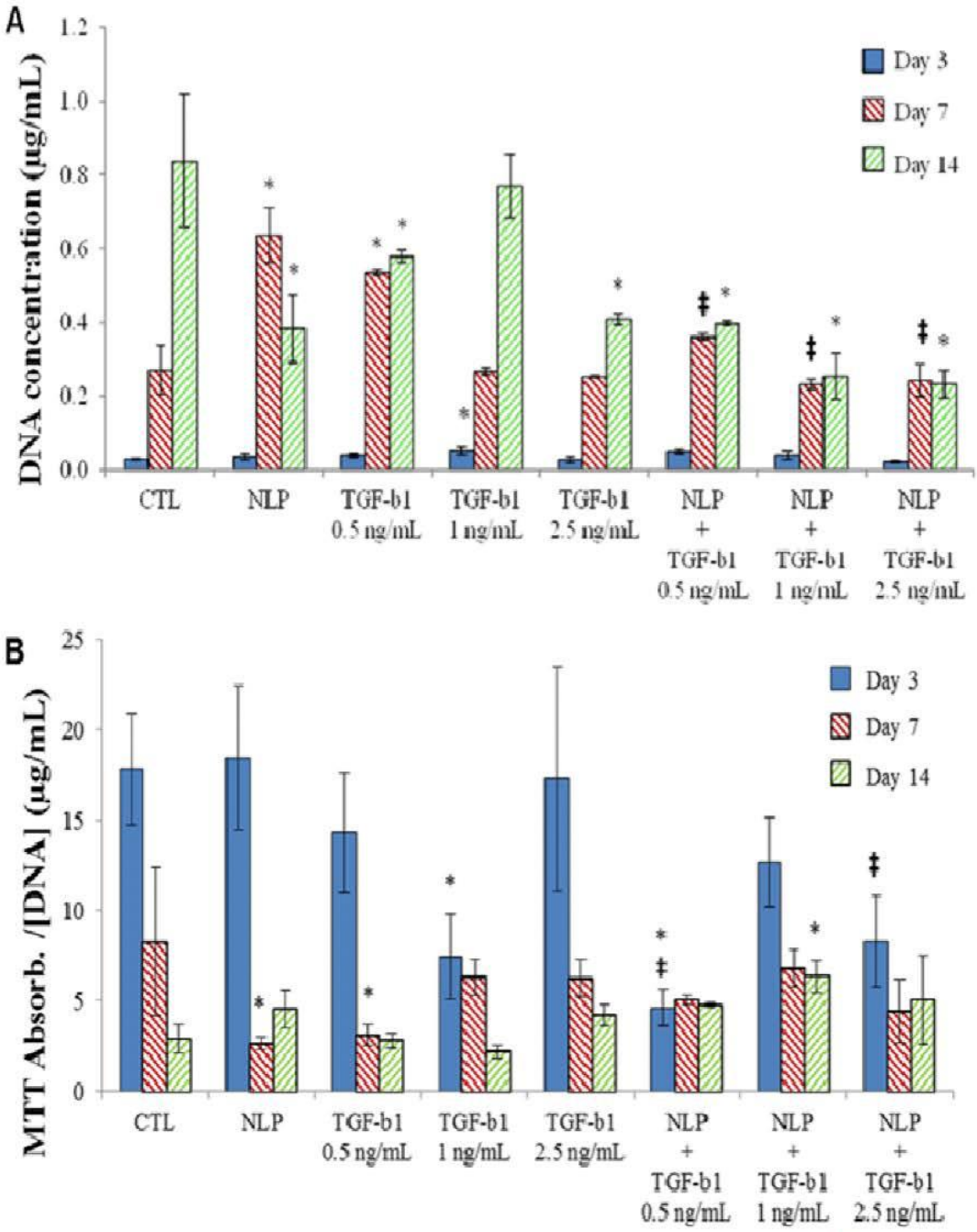

Figure 2. Cell proliferation (A) and metabolic activity (B) of hMSCs cultured with TGF-β1 alone or nanoliposome encapsulated at day 3, 7 and 14. (A) Measurement of DNA. (B) Measurement of MTT absorbance at 550nm and 700nm and normalised by DNA concentration. * Significant difference compared to control group (CTL); ‡ significant difference compared to nanoliposome group (NLP); $p<0.05$

labelling DNA nucleus and F-actin filament of cells (Figure 3) showed normal fusiform shape and assessed a proliferation during cell culture with different proliferation rate depending on the composition of the medium.

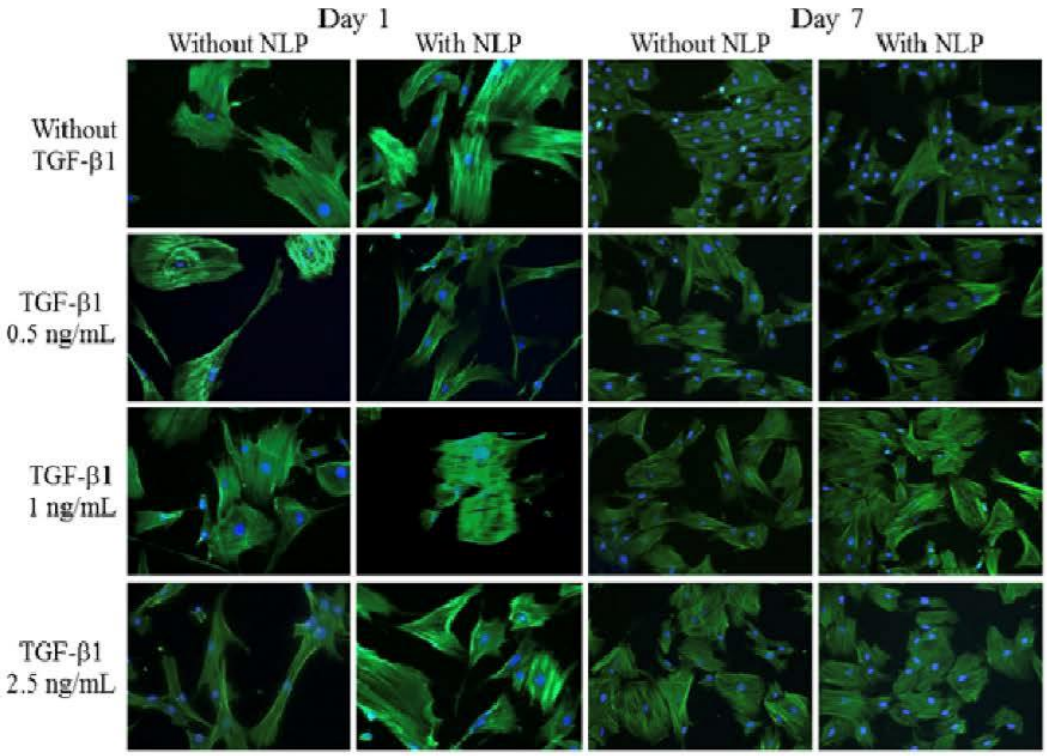

Figure 3. Cell shape and proliferation visualisations made by F-actin (green) and DNA (blue) immunostaining on confocal microscopy at day 1 and 7 for cell stimulation with TGF-β1 concentration from 0 to 2.5ng/mL encapsulated or not into nanoliposomes (NLP).

CONCLUSIONS

During this study, the feasibility of TGF-β1 encapsulation within nanoliposome made of marine lecithin was highlighted. *In vitro* biocompatibility test showed that neither nanoliposome alone nor nanoliposome functionalized with TGF-β1 were cytotoxic on hMSCs at the tested concentrations. Indeed, according to time, no cell under the different stimulations showed differences in term of shape. Nevertheless, TGF-β1 has already shown cell mediation property for MSC differentiation on other cell types and is well known to be involved in many cellular mechanisms (Darling and Athanasiou, 2005; Johnstone et al., 1998; Yang et al., 2011). Thus, the decrease of cell metabolic activity observed might be attributed to TGF-β1 differentiation processes on stem cells. Despite of decreasing cell metabolism activity, cells showed a proliferation rate comparable to cell control group under standard cell culture conditions. This study represents a first experimental approach based on cell morphology and biocompatibility which showed no *in vitro* negative effect on cells. It will be followed with protein and transcriptomic analyses, which will precise hMSCs response to TGF-β1 nanoliposome encapsulated in time and dose dependency. Finally, these initial results are encouraging and constitute promise for future in the field of medical treatment. Indeed, to develop new therapies, vectors reveal themselves more and more reliable when biological and chemical compounds transportation, preservation and release are needed. Nanoliposomes show a great potential in this field by their high biocompatibility and availability and their possibility to encapsulate either hydrophobic or hydrophilic compounds respectively inside lipids layers or interlayer gaps making of it a promising candidate as a compound vector. Moreover, in order to specify the location of compound release, the addition of mediator molecules at the outer phospholipidic layer could be done to increase the efficiency of stimulations by targeting appropriate tissues and cells. TGF-β1 showed a potential to differentiate or is involved in MSC *in vitro* differentiation into several cell types such as smooth muscle cells (SMCs) or chondro

cytes (Johnstone et al., 1998; Yang et al., 2011). Thus, it is of interest to analyse gene expression of SMC or chondrocyte characteristic proteins when exposed to nanoliposomes and to different concentration of TGF-β1 nanoliposome encapsulated. This work highlighted the possibility to use nanoliposomes as nanocarriers for the transport of biological entity. Nanoliposomes could be used to transport and preserve biological active molecules in order to carry biomolecules in specific sites without denaturation. In this case, nanoliposome toxicity has to be tested with a broad-range of cell type before being used *in vivo*. This work has already been begun by testing nanoliposomes on mesenchymal stem cells (rat bone marrow stem cells (Arab Tehrany et al., 2012) and human Wharton's jelly stem cells), cancer cells (MCF7). This work will be continued on macrophages and immune cells.

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Résumé : Les pathologies musculo-squelettiques affectant les os et les articulations demeurent un défi pour la médecine régénératrice. Les difficultés retrouvées sont liées aux besoins d'une vascularisation tissulaire optimale pour les substituts osseux et à l'obtention d'un cartilage de qualité pour les substituts articulaires. Les objectifs de ces travaux de thèse étaient de parvenir à différencier des cellules souches mésenchymateuses (CSM) ombilicales humaines – outil de choix en médecine régénérative - vers un phénotype vasculaire et un phénotype chondrogénique articulaire pour répondre aux besoins de l'ingénierie tissulaire musculo-squelettique. La différenciation de CSM ombilicales en cellules musculaires lisses vasculaires a été montrée après 12 jours de stimulation par des facteurs solubles comme le transforming growth factor (TGF)-beta1 et l'acide ascorbique. Face aux limites des supports de culture en deux dimensions in vitro, un modèle de culture tridimensionnel a été mis en place à l'aide de CSM cultivées en "pellets" en présence ou non d'une solution matrice extracellulaire (MEC) ombilicale pour les expériences suivantes. Les facteurs solubles disposant d'une efficacité limitée et étant souvent onéreux, une transduction des "pellets" de CSM-GW a été réalisée à l'aide de virus adéno-associés recombinants (rAAV) permettant une synthèse de facteurs par les cellules à plus long terme. Ici, les pellets ont été transduits à l'aide de rAAV contenant le gène du facteur de transcription Sox9 afin d'induire une différenciation chondrogénique articulaire. Ces travaux démontrent l'intérêt d'associer la MEC et les CSM ombilicales humaines dans un modèle de culture en trois dimensions, ainsi que les outils de thérapie génique comme les rAAV pour constituer des implants utilisables pour régénérer les tissus musculo-squelettiques.
Mots-clefs: ingénierie tissulaire; cellules souche; matrice extracellulaire; différenciation; rAAV

Abstract : Musculoskeletal conditions affecting bones and joints remain a challenge for regenerative medicine. The difficulties found are related to the needs of an optimal tissue vascularization for bone substitutes and to obtain a cartilage of quality for articular substitutes. The objectives of this PhD work were to differentiate human umbilical mesenchymal stem cells (MSC) - a tool of choice in regenerative medicine - towards a vascular phenotype and a joint chondrogenic phenotype to meet the needs of musculoskeletal tissue engineering. The differentiation of umbilical MSC into vascular smooth muscle cells was shown after 12 days of stimulation by soluble factors such as transforming growth factor (TGF)-beta1 and ascorbic acid. Facing the limits of two-dimensional culture supports in vitro, a three-dimensional culture model was set up using MSC cultured in pellets with or whitout an umbilical extracellular matrix (ECM) solution for the following experiences. Soluble factors have a limited efficacy and are often expensive, a transduction of MSC pellets was performed using recombinant adeno-associated viruses (rAAV) allowing a long term synthesis of factors by the cells. Here, the pellets were transduced using rAAV containing the Sox9 transcription factor gene to induce articular chondrogenic differentiation. This work demonstrates the interest of associating ECM and human umbilical MSC in a three-dimensional culture model, as well as gene therapy tools such as rAAVs to provide grafts that can be used to regenerate musculoskeletal tissues.
Keywords: tissue engineering; stem cells; extracellular matrix; differentiation; rAAV

Zusammenfassung : Muskel- und Skeletterkrankungen an Knochen und Gelenken bleiben eine Herausforderung für die regenerative Medizin. Die Schwierigkeiten stehen im Zusammenhang mit den Ansprüchen einer optimalen Gewebevaskularisierung der Knochenersatzstoffe und damit, einen qualitativ hochwertigen Knorpel für den Gelenkersatz zu erhalten. Das Ziel dieser Doktorarbeit war es, humane mesenchymale Stammzellen (MSZ) aus der Nabelschnur, die das Werkzeug der Wahl in der regenerativen Medizin sind, in einen vaskulären und in einen chondrogenen Phänotyp zu differenzieren, um den Anforderungen des muskuloskeletalen Tissue Engineerings zu entsprechen. Die Differenzierung von Nabelschnur-MSZ in vaskuläre glatte Muskelzellen konnte durch Stimulation mit löslichen Faktoren wie dem Transforming Growth Factor (TGF)-beta1 und Ascorbinsäure nach 12 Tagen gezeigt werden. Angesichts der Grenzen einer zweidimensionalen Zellkultur in vitro, wurde ein dreidimensionales Kulturmodell, in dem MSZ in Pellets mit oder ohne extrazelluläre Matrix der Nabelschnur (EZM) kultiviert wurden, für die weiteren Versuche erstellt. Lösliche Faktoren haben eine limitierte Wirksamkeit und sind häufig teuer. Deshalb wurden die MSZ-Pellets mit rekombinanten Adeno-assoziierte Viren (rAAV) transduziert, was eine Synthese der Faktoren durch die Zellen über einen längeren Zeitraum ermöglicht. In unserem Fall wurden die Pellets mit rAAV, die das Transkriptionsgen Sox9 enthalten, tranzduziert, um eine artikuläre chondrogene Differenzierung zu induzieren. Diese Arbeit zeigt, dass es von Interesse ist EZMs und humane Nabelschnur-MSZ in einem dreidimensionalen Kulturmodell zu vereinen. Auch wird gezeigt, dass Werkzeuge der Gentherapie wie rAAVs, die den Transplantaten zugeführt werden, helfen können muskuloskeletales Gewebe zu regenerieren.
Schlüsselwörter: Tissue Engineering; Stammzellen; extrazelluläre Matrix; Differenzierung; rAAV