Conception et développement d'hydrogels pour l'ingénierie tissulaire appliquée au tissu osseux

Mathieu Maisani

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THÈSE EN COTUTELLE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

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L'UNIVERSITÉ DE BORDEAUX

ET DE L'UNIVERSITÉ LAVAL

ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTÉ

DÉPARTEMENT DE GÉNIE DES MINES, DE LA MÉTALLURGIE ET DES MATÉRIAUX
Interface Chimie-Biologie

Par Mathieu MAISANI

CONCEPTION ET DÉVELOPPEMENT D'HYDROGELS POUR L'INGÉNIERIE TISSULAIRE APPLIQUÉE AU TISSU OSSEUX

Sous la direction de Olivier CHASSANDE
Et de Diego MANTOVANI

Soutenue le 22 septembre 2017

Membres du jury :

Mme. CHAUSSAIN, Catherine, PU-PH, Université Paris Descartes	Président
Mme. LEVISAGE, Catherine, DR INSERM, Université de Nantes	Rapporteur
M. FORTIN, Thomas, MCU-PH, Université de Lyon	Rapporteur
Mme. CHAUSSAIN, Catherine, PU-PH, Université Paris Descartes	Examinateur
M. LAROCHE, Gaétan, Professeur des universités, Université Laval	Examinateur
M. FRICAIN, Jean-Christophe, PU-PH, Université de Bordeaux	Invité

Titre : conception et développement d'hydrogels pour l'ingénierie tissulaire appliquée au tissu osseux

Résumé : Le besoin clinique de nouvelles stratégies pour pallier aux limites des techniques actuelles dans le cas de régénération osseuse a permis l'émergence de l'ingénierie tissulaire osseuse. Les stratégies basées les techniques d'ingénierie tissulaire semblent être une alternative à l'utilisation de greffes et ainsi de s'affranchir des limites qu'elles présentent. L'approche adoptée dans le cadre de cette thèse consiste en le développement et l'utilisation d'hydrogels comme matériaux d'échafaudage pour le comblement et la régénération de tissus osseux. De nombreuses approches utilisant elles aussi des hydrogels existent, chacune possède ses avantages et limites. Dans ce contexte, nos travaux ont consisté en l'utilisation d'un hydrogel non-polymérique comme matériau de base dans le développement des stratégies. Brièvement, plusieurs types cellulaires sont présents au sein du tissu osseux et vont participer aux processus de formation et de régénération osseuse. L'objectif de nos stratégies a été l'apport de cellules souches exogènes puis leur différenciation en cellules ostéoformatrices, ou le recrutement et la différenciation des cellules de l'hôte en cellules ostéoformatrices. Le gel de GNF a été utilisé comme matrice tridimensionnelle pour ses propriétés d'injectabilité, de gélification en l'absence d'agent de réticulation toxique et son potentiel ostéoinducteur. Ce travail a consisté au développement de stratégies pour l'ingénierie tissulaire osseuse en associant le gel de GNF à une matrice naturelle de collagène cellularisée ou à des molécules bioactives pour promouvoir la régénération de lésions osseuses. Ces travaux ont permis de développer et caractériser des stratégies pertinentes pour la régénération de lésions osseuses basées sur l'utilisation d'hydrogels.

Mots clés : Ingénierie tissulaire ; Os ; Hydrogel

Title: Design and development of hydrogels for bone tissue engineering

Abstract: New strategies to overcome the clinical limitations of current techniques for bone defect filling and regeneration has led to the involvement of bone tissue engineering. Indeed, strategies based on tissue engineering techniques seem to be an alternative to the use of grafts and thus to defeat their limits. The approach employed in this thesis consists in development and use of hydrogels as scaffold materials for bone defect filling and regeneration. There are many approaches that also use hydrogels, each one with its advantages and limitations. In this context, our work consisted in the use of a non-polymeric hydrogel as basic material in the development of strategies for bone tissue engineering. Briefly, several cell types are present within bone tissue and will participate in the processes of bone formation and regeneration. The objective of our strategies was the contribution of exogenous stem cells and then their differentiation into osteogenic cells or the recruitment and differentiation of the host cells into osteogenic cells within the material. The GNF gel was used as a three-dimensional matrix considering its properties of injectability, gelation in the absence of toxic crosslinking agent and its osteoinductive potential. The goal was to develop strategies for bone tissue engineering by combining the GNF gel with a natural matrix of cellular collagen or bioactive molecules to promote the regeneration of bone lesions. This work allowed to develop and characterize strategies relevant to the regeneration of bone lesions based on the use of hydrogels.

Keywords : Tissue engineering ; Bone ; Hydrogel

Unités de recherche

Laboratoire BioTis, INSERM U1026, 146 rue Léo Saignat, 33076 Bordeaux, France

Laboratoire de Biomatériaux et de Bioingénierie, Centre de recherche du CHU de Québec,10 rue de l'Espinay, G1L3L5 Québec, Canada

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Liste des abréviations

ALP : Alkaline Phosphatase ou phosphatase alcaline
ARIF : Activation, Résorption, Inversion, Formation
ASCs ou **hASCs** : Adipose Derived Stem Cells ou cellules souches dérivées du tissu adipeux (human ou humaines)
BMPs : Bone morphogenetic protein ou protéines morphogéniques osseuses
BMSC : Bone Marrow Stromal Cells ou Cellules stromales dérivées de la moelle osseuse
BMU : Basic Multicellular Unit ou Unité Multicellulaires Basique
BTE : Bone Tissue Engineering
DMEM : Dulbecco's Modified Eagle's Medium
DPSCs : Dental Pulp Stem Cells ou Cellules Souches issues de la Pulpe Dentaire
ECM : Extracellular Matrix
ECP : Endothelial Progenitor Cells
ESC : Embryonic Stem Cells
ESHU : poly(ethylene glycol)–poly(serinol hexamethylene urethane)
FACIT : Fibril Associated Collagen with Interupted Triple helixes ou collagènes associés à des fibrilles avec des triples hélices interrompues
FBS : Fetal Bovine Serum
FBs : Fibroblasts
FDA : Food and Drug Administration
FGF : Fibroblast Growth Factor ou facteurs de croissance des fibroblastes
FT-IR : Fourier Transform InfraRed spectroscopy ou spectroscopie infrarouge à transformée de Fourier
GAG : Glycosaminoglycanes
GFP : Green Fluorescent protein
GNF : Glycosil-Nucléoside-Fluoré
GNL : Glycosyl-Nucléo-Lipides
HA : Hydroxyapatite
HEPES : (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSCs : Hematopoietic Stem Cells ou Cellules Souches Hématopoïétiques
HUVEC : endothelial cells isolated from umbilical cord vein
IGF : Insuline-like Growth Factor ou facteur de croissance 1 ressemblant à l'insuline
IL : Interleukine
iPSCs : induced Pluripotent Stem Cells
ITO : Ingénierie du Tissu Osseux
LCST : Lower Critical Solution Temperature ou température critique inférieure de solubilité
M-CSF : Macrophage colony-stimulating factor ou facteur de stimulation des colonies de macrophages
MEC : Matrice Extra Cellulaire
MPEG-PCL : Methoxy-PEG-*b*-polycaprolactone
MSCs : Mesenchymal Stem Cells ou Cellules Souches Mésenchymateuses
ODF : Osteoclast Differentiation Factor ou facteur de différenciation des ostéoclastes
PAL : Phosphatase Alcaline
PCL : Polycaprolactones
PDGF : Platelet-derived growth factor ou facteur de croissance dérivé des plaquettes
PEEK: Polyetheretherketone
PEG : Polyethylenglycol
PEGDA: Diacrylate PEG
PEO : Polyethylene oxide

PFA : Paraformaldehyde
PGs : Protéoglycanes
PNIPAAm : Poly(N-isopropylacrylamide)
PPE : Polyphosphoester
PTH : Pathyroid Hormone ou Parathormone
RANKL : receptor activator of nuclear factor kappa-B ligand ou récepteur du ligand Kappa-B du facteur nucléaire
RGD : Tripeptide Arg-Gly-Asp
SMCs : Smooth Muscle Cells
TEPs : Tissue Engineering Products
TGF-β : Transforming Growth Factor ou facteurs de croissance transformant
TNF-α : tumor necrosis factor ou facteur de nécrose tumorale
VEGF : Vascular endothelial growth factor ou facteur de croissance de l'endothélium vasculaire

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À Joseph.

Avant-Propos

Chaque année, des millions d'interventions chirurgicales sont réalisées dans le monde entier pour le traitement de lésions osseuses. Bien que le tissu osseux ait la capacité de se réparer naturellement, dans le cas de lésions trop importantes ou de certaines pathologies ce potentiel de régénération n'est pas suffisant. Il est alors nécessaire pour le clinicien d'avoir recours à l'utilisation d'autogreffes, d'allogreffes ou de biomatériaux exogènes. Toutefois ces approches présentent de nombreuses limitations. C'est dans ce contexte que l'ingénierie tissulaire osseuse semble être une approche prometteuse pour favoriser la régénération des tissus osseux en proposant une alternative aux techniques actuelles. Cette approche repose sur l'utilisation de biomatériaux comme matrice tridimensionnelle (ou échafaudage) pour favoriser et guider la formation de nouveaux tissus osseux. Les hydrogels sont parmi les biomatériaux les plus prometteurs pour une application en ingénierie tissulaire osseuse. En effet leurs propriétés peuvent leur permettre d'être injectés, minimisant le caractère invasif du geste chirurgical, d'épouser parfaitement la géométrie de la lésion osseuse à combler et de contenir du matériel biologique (cellules, gènes, protéines). Dans ce contexte, l'objectif de cette thèse est de concevoir, développer et étudier de nouvelles stratégies d'ingénierie tissulaire à partir d'hydrogel pour le combement et la régénération du tissu osseux. Pour y parvenir, le premier objectif consiste au développement d'un hydrogel composite adapté à l'injection de cellules souches pour la régénération osseuse. Un second objectif est le développement d'un système de libération de facteurs de croissance, injectable et capable de promouvoir la réparation d'une lésion osseuse.

Ce projet a été mené dans le cadre d'une cotutelle de thèse entre le laboratoire de Biomatériaux et Bioingénierie de l'université Laval sous la direction du Professeur Diego Mantovani et le laboratoire Biotis, sous la direction du Docteur Olivier Chassande.

Dans le cadre de cette cotutelle l'extraction et la réalisation des gels de collagène ainsi qu'une partie de la caractérisation des matériaux ont été réalisés au laboratoire de Biomatériaux et Bioingénierie à Québec. Le développement des hydrogels synthétiques et les études *in vivo* ont été réalisées aux laboratoires de l'université de Bordeaux.

L'introduction de ce manuscrit présente la structure de la thèse, la physiologie du tissu osseux, ses pathologies, les solutions cliniques actuelles et leurs limites. Les concepts d'ingénierie tissulaire et ingénierie tissulaire appliquée au tissu osseux seront ensuite définis. Le premier chapitre présente une revue de la littérature sur l'utilisation des hydrogels pour l'ingénierie tissulaire osseuse. Ce chapitre a fait l'objet d'une publication

dans « journal of tissue engineering », journal international à comité de lecture. Les chapitres deux et trois présentent la conception et la validation de stratégies d'ingénierie tissulaire osseuse. D'une part pour l'injection de cellules souches ou de facteurs de croissance, à partir d'hydrogels et appliquées à la régénération du tissu osseux. Ces chapitres font l'objet d'articles scientifique de recherche originale et sont actuellement en révision dans des journaux internationaux à comité de lecture.

En tant que premier auteur de ces trois articles, j'ai mené ces travaux de leur conception à la validation en collaboration avec des co-auteurs.

Chapitre 3: Cellularizing hydrogel-based scaffolds to repair bone tissue: How to create a physiologically-relevant microenvironment? Auteurs : Mathieu Maisani, Daniele Pezzoli, Olivier Chassande, Diego Mantovani. Journal : Cet article a été publié dans Journal of Tissue Engineering, June-08-2017. doi : 10.1177/2041731417712073. Pour cet article, j'ai effectué les recherches bibliographiques et leur synthèse ainsi que la rédaction en étroite collaboration avec Daniele Pezzoli. Olivier Chassande et Diego Mantovani ont dirigé les recherches et la rédaction du manuscrit.

Chapitre 4: A new composite hydrogel combining the biological properties of collagen with the mechanical properties of a supramolecular scaffold for bone tissue engineering. Auteurs : Mathieu Maisani, Sophia Ziane, Camille Ehret, Lucie Levesque, Robin Siadous, Jean-François Le Meins, Pascale Chevalier, Philippe Barthélémy, Hugo De Oliveira, Joëlle Amédée, Diego Mantovani, Olivier Chassande. Journal : Cet article est en révision dans Journal of Tissue Engineering and Regenerative Medecine. Pour cet article, les expériences et l'analyse des résultats ont été menées par moi-même et en collaboration avec Sophia Ziane, Camille Ehret qui avaient précédemment travaillé sur ce projet. Lucie Levesque a réalisé les extractions de collagène, Pascale Chevalier les analyses FTIR, Jean-François Le Meins les analyses rhéologiques. J'ai rédigé la majeure partie de l'article. L'ensemble a été corrigé par les auteurs.

Chapitre 5: Prolonged delivery of BMP-2 by a non-polymer hydrogel for bone defect regeneration. Auteurs : Mathieu Maisani, Sindhu, Mathilde Fenelon, Robin Siadous, Sylvie Rey, Diego Mantovani, Olivier Chassande. Journal : Cet article a été soumis à Biomaterials. Dans cet article, j'ai réalisé la conception des hydrogels chargé en BMP-2 et ai fait toutes les expériences. Sindhu KR, Mathilde Fénelon, Robin Siadous et Sylvie Rey m'ont respectivement apporté leur expertise et ont participé à la mesure de fluorescence in vivo, la réalisation des lésions osseuses chez la souris, l'extraction de

cellules souches humaines, la coloration de coupes de tissus fixés. J'ai effectué l'entière rédaction qui a été corrigée et améliorée par Olivier Chassande et Diego Mantovani.

Après les résultats, le dernier chapitre présente une discussion générale sur le travail effectué tout au long de cette thèse. Cette section met en relief et discute les méthodologies et les techniques développées pour réaliser les travaux présentés ainsi que les défis rencontrés et propose les perspectives envisagées.

Structure de la thèse

L'**introduction** de cette thèse traite de la physiologie osseuse ainsi que des mécanismes de guérison du tissus osseux. Sont ensuite présentées les stratégies cliniques de thérapie et de comblement osseux puis les stratégies alternatives aux techniques actuelles comme l'ingénierie tissulaire.

À la suite de l'introduction les **approches et objectifs** de l'étude sont développés.

Le chapitre suivant (**chapitre 3)** est une revue de la littérature publiée dans « Journal of Tissue Engineering » dans le cadre du numéro spécial « Tissue engineering biomimetic micro-environment to direct cell function ». Le manuscrit traite de la création de microenvironnement aux propriétés physiologiques adéquates pour la régénération du tissu osseux à partir d'hydrogels : "Cellularizing hydrogel-based scaffolds to repair bone tissue: How to create a physiologically-relevant microenvironment?". Ce manuscrit présente l'état de l'art concernant les stratégies d'ingénierie tissulaire dédiées à la réparation osseuse. Il traite des nombreux facteurs qui déterminent la survie, la capacité de différenciation et de formation osseuse des différents types de cellules souches humaines utilisées en ingénierie tissulaire. Les limites actuelles à surmonter pour la conception des matrices d'échafaudage pour l'application clinique future de ces matériaux sont exposées. Ainsi ce travail aborde de nombreuses disciplines telles que l'anatomie et la physiologie lors de la description du tissu osseux et de ses mécanismes de réparation. La chimie et la physique sont abordés dans le cadre de la description des différents matériaux d'échafaudage sous forme d'hydrogel. La biochimie intervient quant à elle pour la description des facteurs de croissance et de la biologie cellulaire. Enfin les modèles expérimentaux *in vivo* sont présentés pour l'évaluation de la fonction des stratégies d'ingénierie tissulaire. Compte tenu du grand nombre d'articles publiés dans ce domaine, nous avons limité notre recherche aux publications les plus récentes, en privilégiant les articles présentant des études réalisées *in* vivo. Néanmoins, cette revue cite 254 références, parmi lesquelles des revues récentes qui peuvent aider le lecteur à explorer plus en détail certains des nombreux domaines abordés dans ce manuscrit. Au cours de ce chapitre nous soulignons les limites scientifiques et techniques de ces stratégies qui devront être abordée lors des recherches futures.

Le **chapitre 4** présente une étude expérimentale ayant conduit à la rédaction d'un article scientifique : « A new composite hydrogel combining the biological properties of collagen with the mechanical properties of a supramolecular scaffold for bone tissue engineering. ». Ce travail présente la conception, la validation et la caractérisation d'un

matériau pour l'ingénierie tissulaire osseuse. L'objectif est d'optimiser le choix des éléments constitutifs du produit d'ingénierie tissulaire ainsi que les méthodes de conception de celui-ci et d'en réaliser la caractérisation *in vitro* et *in vivo*. Ainsi ce manuscrit décrit les propriétés d'un hydrogel hybride constitué de collagène associé à un nouveau type d'hydrogel physique non polymérique et son potentiel en tant que matrice d'échafaudage pour les cellules souches dérivées du tissu adipeux (hASCs) en vue de constituer un produit pour l'ingénierie du tissu osseux. Ce chapitre concerne à la fois la caractérisation physico-chimique de ces gels : FT-IR, Rhéologie, microscopie électronique à balayage, ainsi que ses propriétés biologiques : caractérisation *in vitro* et *in vivo* des complexes matériaux-cellules (survie et différenciation des cellules souches ensemencées).

Cet hydrogel composite a montré des caractéristiques mécaniques et biologiques supérieures par rapport aux hydrogels de collagène ou de GNF étudiés séparément. La présence de l'hydrogel physique de GNF a limité le phénomène de compaction du gel de collagène et donc la diffusion des cellules hors de l'échafaudage. L'étude rhéologique des matériaux a souligné que l'hydrogel composite présente un module élastique plus élevé que celui des gels de collagène et de GNF. L'hydrogel composite a permis l'adhésion cellulaire et la prolifération *in vitro*, et la survie cellulaire à long terme *in vivo* là où les cellules ne survivent pas dans les hydrogels de GNF. En outre, il a favorisé la différenciation des hASCs en l'absence de facteurs ostéogéniques. *In vivo*, les cellules ensemencées dans le gel composite et injectées ensite sous-cutanée chez des souris immunodéficientes ont produit du tissu ostéoïde et se sont différenciées en cellules osseuses. Cette étude a conduit au développement d'un nouvel hydrogel composite présentant des propriétés prometteuses en tant qu'échafaudage pour des applications en ingénierie des tissus osseux

Le **chapitre 5** présente également une étude expérimentale ayant conduit à la rédaction d'un article scientifique. Les protéines de la morphogenèse osseuse 2 (BMP-2) sont les facteurs de croissance les plus fréquemment utilisés pour les approches de régénération osseuse en clinique. Généralement, elles sont combinées avec des éponges de collagène : ces dernières sont imbibées d'une solution de facteur de croissance avant l'implantation. Bien que ces protocoles soient cliniquement établis, ces techniques n'offrent qu'un faible contrôle sur la libération des facteurs de croissance. En effet, les BMPs sont rapidement transmises au site lésé et à des concentrations supra-physiologiques, entraînant des effets secondaires indésirables, limitant leur utilisation en clinique. Les travaux réalisés au cours de cette étude ont permis de développer un

système de libération de BMP-2 pour l'ingénierie du tissu osseux. Après avoir étudié les cinétiques de relargage de la BMP-2 contenue dans les hydrogels et son effet sur des cultures cellulaire *in vitro*, les matériaux étaient implantés en lésion osseuse de calvaria chez la souris afin d'étudier la capacité des hydrogels à maintenir la BMP-2 sur le site lésé. Une analyse radiographique (plane et tridimensionnelle) puis histologiques a ensuite réalisée afin d'étudier la capacité des matériaux à promouvoir la régénération osseuse. Nous montrons que la BMP-2 peut être facilement encapsulé dans ces gels et lentement libéré *in vitro*, tout en maintenant son activité ostéogénique. Lorsque l'hydrogel contenant une faible dose de BMP-2 est déposé sur un défaut de calvaria réalisé chez la souris, la protéine est délivrée pendant plusieurs semaines et favorise la régénération osseuse. Lors du sacrifice des animaux, l'os nouvellement formé couvrait la majeure partie de la lésion osseuse. De plus, il présentait une épaisseur et des caractéristiques histologiques similaires à celles de l'os natif. Les travaux menés lors de cette étude ont conduit à la validation de l'hydrogel de GNF comme un système de libération de BMP-2 facile à utiliser et efficace pour la régénération osseuse.

Au cours de la **Discussion générale** puis des **Limites des modèles et outils; perspectives** de l'étude, les résultats et les stratégies présentés au cours des chapitres précédents sont discutés. Nous reviendrons sur le choix des hydrogels, des types cellulaires et des facteurs de croissance utilisés; sur les méthodes de conception des matériaux; sur les techniques d'études des propriétés ostéoinductrices. Enfin, les perspectives pour les travaux futurs sont proposées.

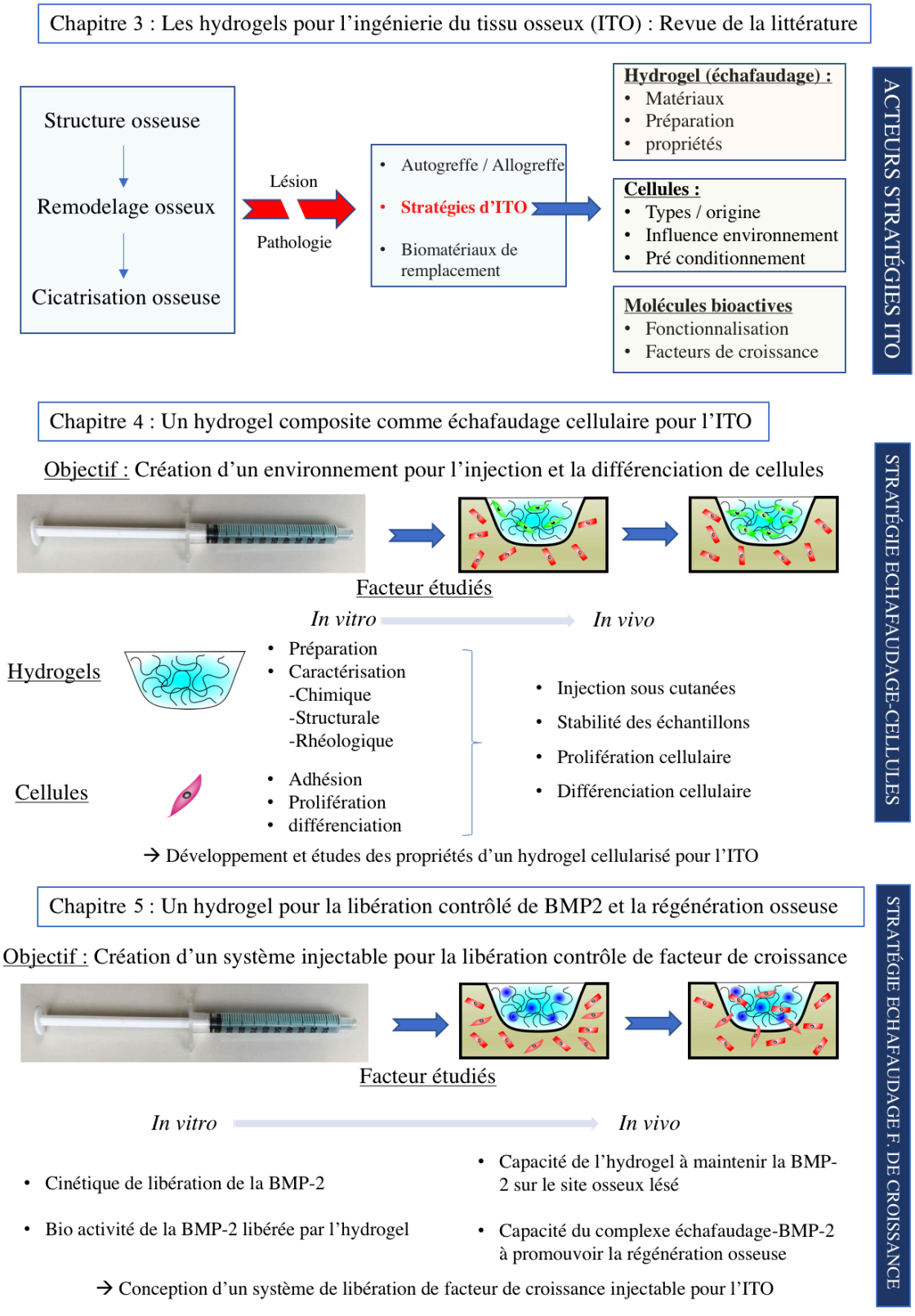

Figure 1 Structure de la thèse

1. Introduction

1.1. Physiologie osseuse et mécanismes de guérison

1.1.1. Définition et fonctions du tissu osseux

Le squelette humain est composé de 206 os : 80 pour l'axe tête-cou-tronc et 126 pour les membres. Ils sont généralement classés sur la base de leur forme, dans quatre catégories principales : i) os longs (par exemple, l'humérus, le radius, l'ulna, le fémur, le tibia), ii) os courts (par exemple, les os du carpe et du tarse), iii) os plats (par exemple, os du crâne, les omoplates, le sternum, les côtes), et iv) os irréguliers (par exemple, les vertèbres, le coccyx, le sacrum)[1].Les différents tissus composant l'os sont les tissus osseux spongieux et compacts, le tissu osseux cartilagineux, le cartilage fibreux (insertion des tendons), la moelle osseuse hématopoïétique (cavité centromédullaire). Le tissu osseux a trois grandes fonctions :

1. Mécanique : il apporte soutien et protection aux organes vitaux et au système nerveux (rôle statique) et il permet de transmettre les forces d'une partie du corps à une autre (rôle dynamique). Les propriétés mécaniques de l'os sont un compromis entre le besoin de raideur (réduction de la contrainte et efficience cinématique) et le besoin de ductilité pour absorber les chocs (réduction du risque de fracture). Pour répondre à ce compromis, des milliers d'années d'évolution ont produit une microstructure complexe, anisotrope, multiphasique et hétérogène, qui est décrite ci-après.

2. Métabolique : le tissu osseux est un tissu dynamique en renouvellement permanent sous l'effet des contraintes mécaniques. Ce remodelage entraine le stockage ou la libération de sels minéraux. L'os participe ainsi avec l'intestin et les reins à l'homéostasie phosphocalcique de l'organisme.

3. Hématopoïétique : la moelle hématopoïétique, renfermée dans l'espace médullaire de l'os est à l'origine de la production des trois types de lignées de cellules du sang.

1.1.2. Structure osseuse

L'os est un tissu conjonctif qui peut être considéré comme un matériau cellularisé composite, la partie interne est composée d'un os spongieux, souvent appelé os trabéculaire, alors que la parie externe est formée par un os compact aussi défini comme os cortical. Le ratio de masse relative entre ces deux types d'os varie de 20 % à 80 % dans l'ensemble du système squelettique, en fonction des os concernés. La principale différence entre les deux types d'os réside dans leur porosité qui peut varier fortement[2].

1.1.2.1. L’os trabéculaire ou spongieux

Le premier type est l’os trabéculaire ou spongieux présentant 30 à 95 % de porosité, habituellement trouvé dans les os cubiques, les os plats et aux extrémités des os longs. Cette structure poreuse est remplie de moelle osseuse qui est constituée de vaisseaux sanguins, de nerfs et de divers types cellulaires, responsable de l’hématopoïèse ainsi que les ostéocytes trabéculaires qui reçoivent des éléments nutritifs à travers le réseau canaliculaire. La matrice est formée par un réseau tridimensionnel de trabécules, ramifiées et anastomosées avec une épaisseur d'environ 100 à 150 µm et un espace entre les travées de 500 à 1000 µm. Leur agencement est variable. Les trabécules ont une organisation lamellaire similaire à celle des ostéons, mais sans canal interne et sans vascularisation.

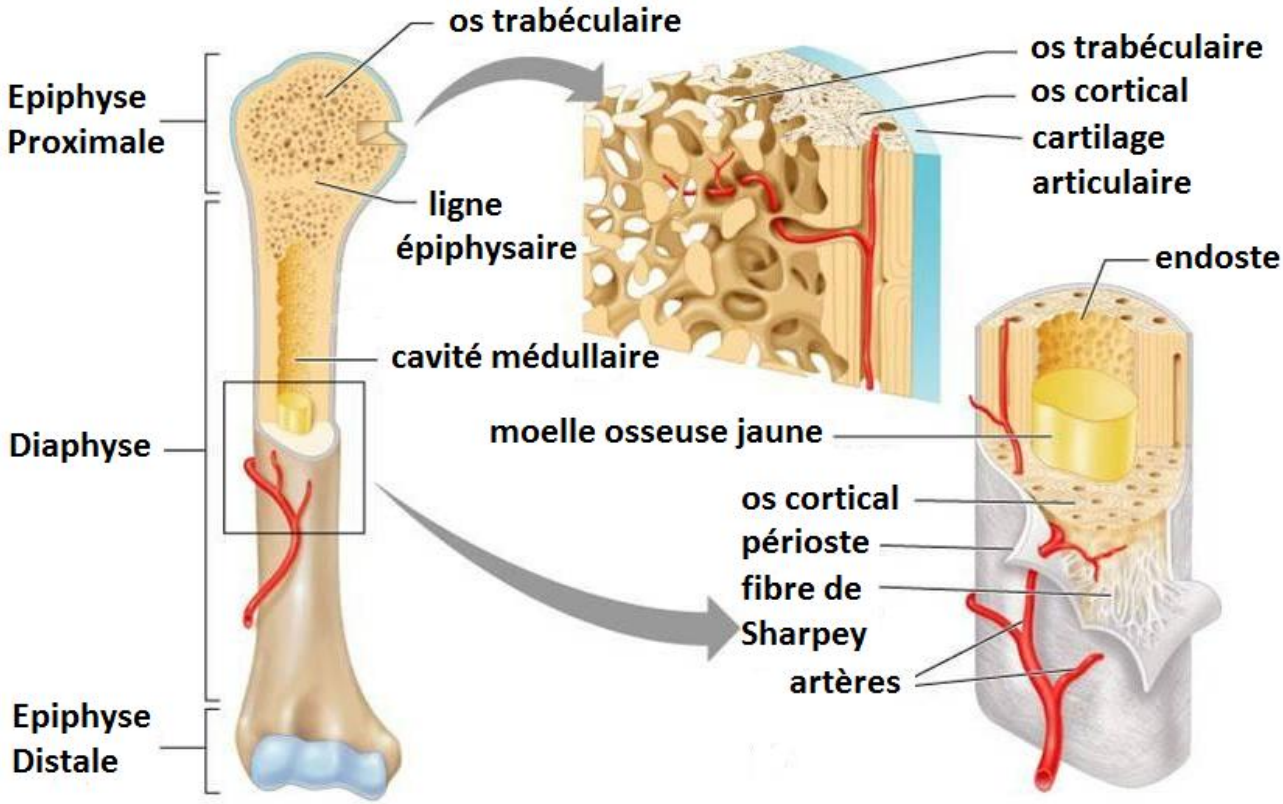

Figure 2 : Structure et organisation des os longs [3]

1.1.2.2. L'os cortical

En opposition, l'os cortical également nommé haversien ou compact est dense et solide, avec une porosité comprise entre 5% et 30% et différents types de pores [4].

On pourra décrire deux sous-unités : la première dite lamellaire où la porosité est la plus faible. La matrice osseuse est disposée en lamelles superposées et les microfibrilles de collagène sont organisées parallèlement selon une direction qui se modifie entre chaque lamelle consécutive de sorte à offrir la meilleure réponse aux contraintes mécaniques. La seconde sous-unité se compose de structures cylindriques appelées ostéons ou systèmes haversiens, avec un diamètre d'environ 200 nm, formée par des lamelles cylindriques entourant le canal de Havers. Cette plus grande porosité est formée par les canaux de Havers (alignés selon l'axe long de l'os) et les canaux de Volkmanns (transversaux, reliant les canaux de Havers) contenant des capillaires et des nerfs. Des lacunes sont présentes, chacune contenant un ostéocyte. De plus, un réseau de petits canaux (canalicules) relie les lacunes et le canal haversien, ce qui permet des interactions cellule-cellule et favorise l'échange de nutriments et de métabolites.

L'os cortical se trouve dans la diaphyse des os longs et sur le pourtour de l'os trabéculaire formant l'enveloppe externe des os plats. Cette combinaison de l'os cortical

et de l'os trabéculaire forme une structure de type «sandwich», reconnu dans l'ingénierie des matériaux pour ses propriétés mécaniques optimales [5].

Au niveau ultra structural, le tissu osseux est composé de matrice extracellulaire (MEC) osseuse et de cellules osseuses.

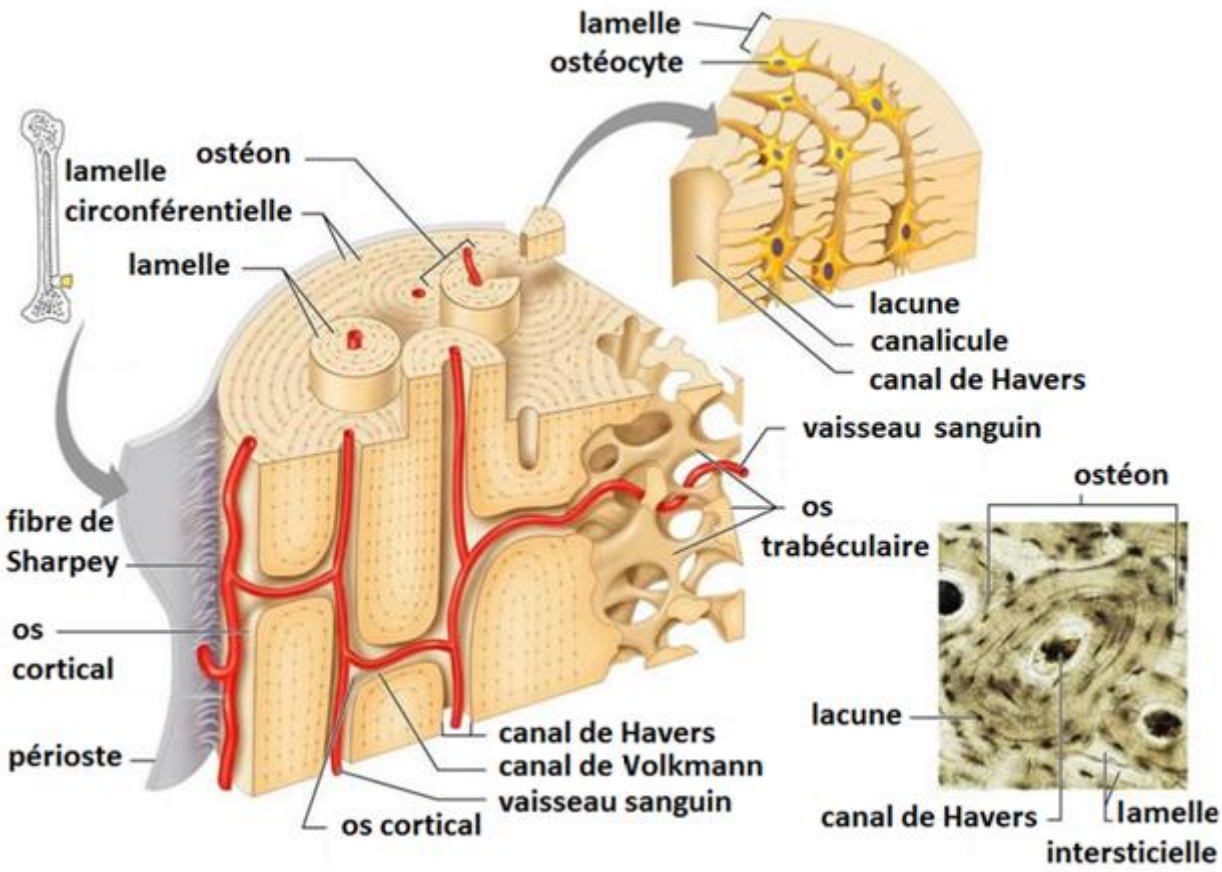

Figure 3 : Organisation et composition de l'os cortical [3]

1.1.2.3. Matrice extracellulaire osseuse

La MEC osseuse se caractérise par deux phases : une phase minérale inorganique et une phase organique composée de protéines.

1.1.2.3.1. La phase inorganique

La phase inorganique est principalement composée de phosphate de calcium qui forme les cristaux d'hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] d'un maximum de 200 nm de long et 20 nm de large. Le carbonate de calcium, le fluorure de calcium et le fluorure de magnésium sont présents au sein de cette phase qui sert de réservoir pour l'homéostasie des ions (calcium, phosphate…). La MEC inorganique apporte au matériau qu'est l'os des propriétés mécaniques importantes. En effet, l'orientation des cristaux d'HA,

généralement selon la direction longitudinale dans les os longs, confère à l'os sa rigidité et sa résistance aux forces de compression.

1.1.2.3.2. La phase organique

La phase organique est principalement composée d'un réseau de collagène de type I (environ 90%), la partie restante étant des protéines non collagéniques telles que les glycoprotéines, les protéoglycanes et des facteurs de croissance. Le rôle structurel de la MEC organique est double : la régulation de la forme de l'os et l'apport de propriétés mécaniques telles que la ductilité et la résistance à la fracture. En outre, la MEC organique est un réservoir de facteurs de croissance et de cytokines impliqués dans le processus de remodelage osseux.

1.1.2.3.3. Le réseau de collagène

Les fibres de collagène présentes dans l'os régulent la nucléation et l'orientation spatiale des cristaux d'HA. En effet, les trois hélices de collagène sont alignées dans les fibrilles de collagène, avec un décalage de 40 nm entre les extrémités de deux sous-unités consécutives. Ces espaces créés sont les sites de nucléation pour les cristaux d'HA dont la taille et l'orientation sont ainsi contrôlées par la structure et l'organisation des fibrilles de collagène[6]. D'autres types de collagènes comme les collagène de type III et V ainsi que les collagènes FACIT (Fibril Associated Collagen with Interrupted Triple helixes) sont présents en quantités infimes et sont rapportés comme ayant un rôle dans la régulation de la fibrillogenèse et dans la modulation du diamètre de fibrille[7,8].

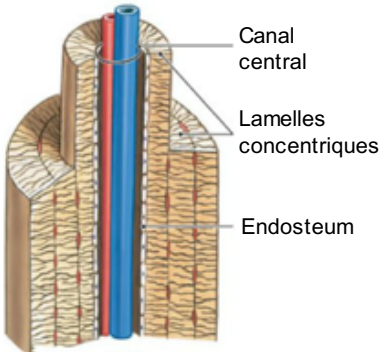

Figure 4 : Ostéon en coupe (adaptée)[9]

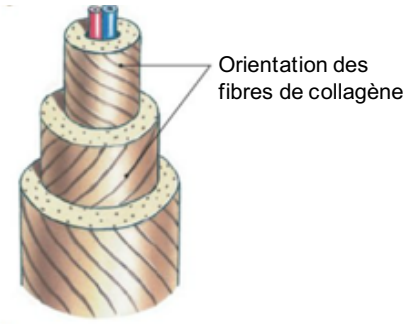

Figure 5 Orientation des fibres de collagène dans les lamelles adjacentes des ostéons (adaptée)[9]

1.1.2.3.4. Les protéines non collagéniques

Parmi les protéines non collagéniques, environ 10% sont constituées de protéoglycanes (PGs) qui résultent de la combinaison d'une protéine et d'un glycosaminoglycane (GAG)[10]. Grâce aux propriétés physico-chimiques de leurs GAGs, les PGs forment une matrice de type « gel » hautement hydratée dont le rôle principal est de fournir une résistance aux contraintes de compressionen agissant comme un amortisseur. D'un point de vue biologique les PGs jouent un rôle important dans la liaison et le stockage des facteurs de croissances ainsi que dans la régulation de la fibrillogenèse du collagène[11].Les glycoprotéines de la MEC osseuses comprennent la phosphatase alcaline (PAL), l'ostéopontine, la sialoprotéine osseuse et l'ostéocalcine, qui sont toutes les trois impliquées dans le processus de minéralisation et l'ostéonectine qui l'est dans la modulation du diamètre des fibrilles de collagène.La fibronectine, la vitronectine, les thrombospondines et les fibrillines qui contiennent les domaines de liaison à l'intégrine RGD participent pour leur part à la connexion entre phases inorganique-organique, aux interactions cellules-matrices et à la limitation de l'élasticité de la MEC osseuse[12].

1.1.3. Les cellules osseuses

Les cellules présentes dans le tissu osseux proviennent de deux types de cellules progénitrices : les cellules souches mésenchymateuses (MSCs) et des cellules souches hématopoïétiques (HSCs). Les cellules osseuses interviennent de façon séquentielle

dans les processus de régénération osseuse selon le cycle ARIF (Activation, Résorption, Inversion, Formation).

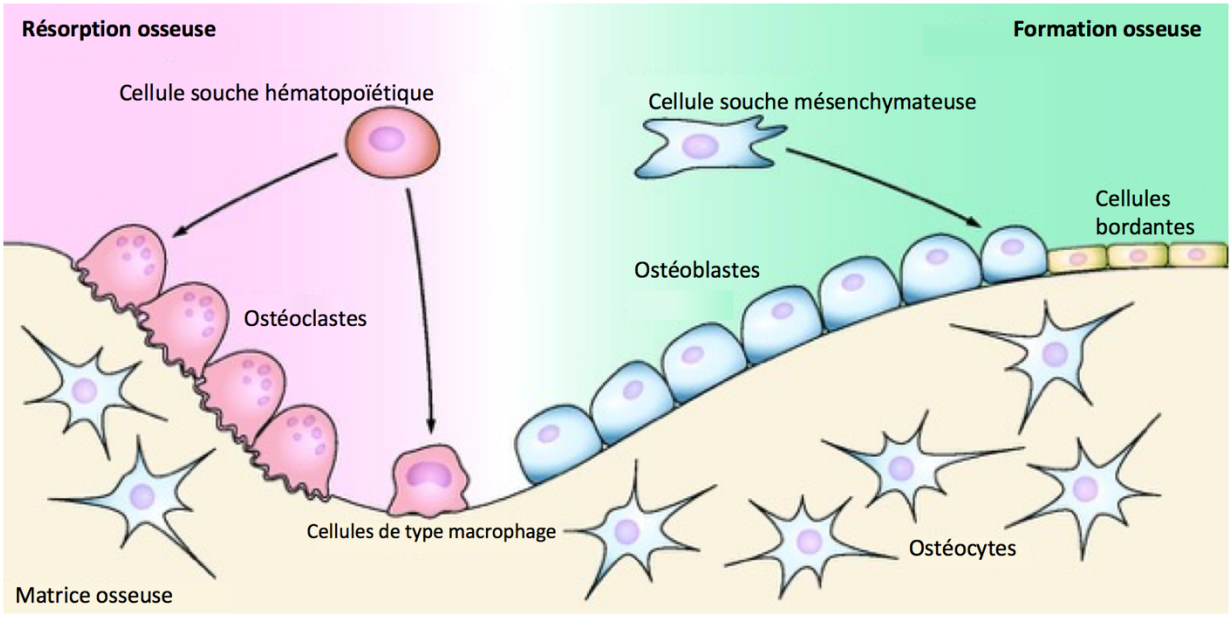

Figure 6 : Différents types de cellules osseuses et leur rôle au sein du cycle de remodelage osseux (adaptée)[13]

1.1.3.1. Les ostéoblastes

Les ostéoblastes sont responsables de la synthèse de la nouvelle MEC osseuse participant ainsi à la formation du tissu osseux. Ils proviennent de MSCs situées dans la cavité médullaire ou dans le périoste qui une membrane fibreuse couvrant la surface externe des os. La différenciation des cellules ostéoprogénitrices en ostéoblastes est induite et régulée par des facteurs de croissance de la super famille des facteurs de croissance transformant (TGF-β) tels que les protéines morphogéniques osseuses (BMPs). L'étape suivante concerne la migration et la prolifération dans un site spécifique (Unité Multicellulaires Basique ou BMU) où les ostéoblastes déposent une MEC organique riche en collagène de type I (ostéoïde) et induisent ensuite la minéralisation de cette matrice[14]. Lorsque les ostéoblastes finissent par déposer une nouvelle matrice, trois principaux destins sont possibles : i) ils restent incorporés dans le tissu minéralisé nouvellement formé et se transforment en ostéocytes ; ii) ils deviennent des cellules bordantes ; iii) ils subissent une apoptose (pour 50 à 70% des cellules).

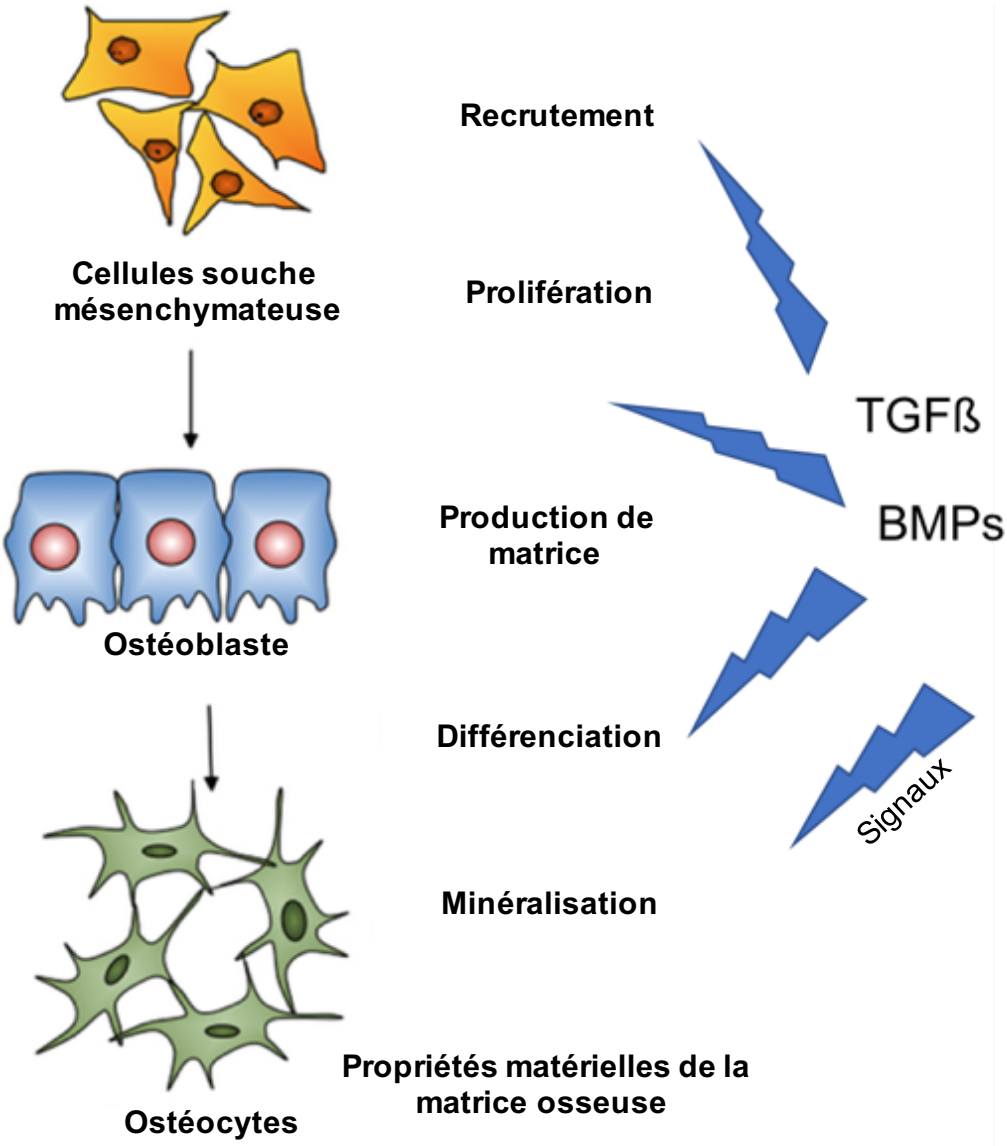

Figure 7 : Cascade de différenciation des ostéoblastes (adaptée)[15]

1.1.3.2. Les ostéocytes

Les ostéocytes sont des ostéoblastes quiescents ayant une faible fonction métabolique. Ils sont situés dans les lacunes osseuses et reliés entre eux par le biais du réseau de canalicules. Le rôle principal des ostéocytes est de réguler le maintien du tissu osseux. En effet, ils servent de capteurs de contrainte et de déformations : en réponse à des stimuli mécaniques, ils peuvent exprimer des signaux paracrines qui régulent l'activité des ostéoblastes actifs et des ostéoclastes, ce qui régit le renouvellement osseux[16].

1.1.3.3. Les cellules bordantes

Les cellules bordantes sont des ostéoblastes quiescents avec une morphologie plate qui sont localisés à la surface de l'os dans des zones non-actives c'est-à-dire ni en formation, ni en résorption[17]. Les cellules bordantes sont interconnectées via des

jonctions lacunaires et communiquent avec d'autres cellules osseuses à travers le réseau de canalicules, formant ainsi avec les ostéocytes et les ostéoblastes un syncytium responsable de la mécano-sensation. Les cellules bordantes forment également une interface entre les vaisseaux sanguin et le tissu osseux qui régule les flux (en particulier ioniques). Elles sont pourvues de récepteurs aux facteurs de croissance ce qui leur permet d'être stimulées pour proliférer et de se redifférencier en ostéoblastes[18].

1.1.3.4. Les ostéoclastes

Les ostéoclastes sont responsables de la résorption des os lors des phases de remodelage. Ils dérivent des précurseurs mononucléaires des cellules de la lignée monocytaires (ayant pour origine les HSCs). Les cellules stromales de la moelle et les ostéoblastes produisent des cytokines, dont l'activateur du récepteur du ligand Kappa-B du facteur nucléaire (RANKL, également connu sous le nom de facteur de différenciation des ostéoclastes (ODF) et le facteur de stimulation des colonies de macrophages (M-CSF). Ils entrainent une fusion des cellules de la lignée monocytaire en grandes cellules multi-nucléées[19]. La résorption osseuse est obtenue par la sécrétion d'ions H^+ par des pompes à protons de la membrane. L'environnement acide ainsi créé (pH = 4,5) dans la lacune de résorption (lacune de Howship) dissout le composant minéral de la matrice tandis que la cathepsine K et d'autres enzymes sont sécrétées afin de dégrader la MEC organique. D'un point de vue morphologique les ostéoclastes possèdent une membrane cytoplasmique fortement ondulée ce qui augmente considérablement la surface cellulaire responsable de la résorption osseuse.

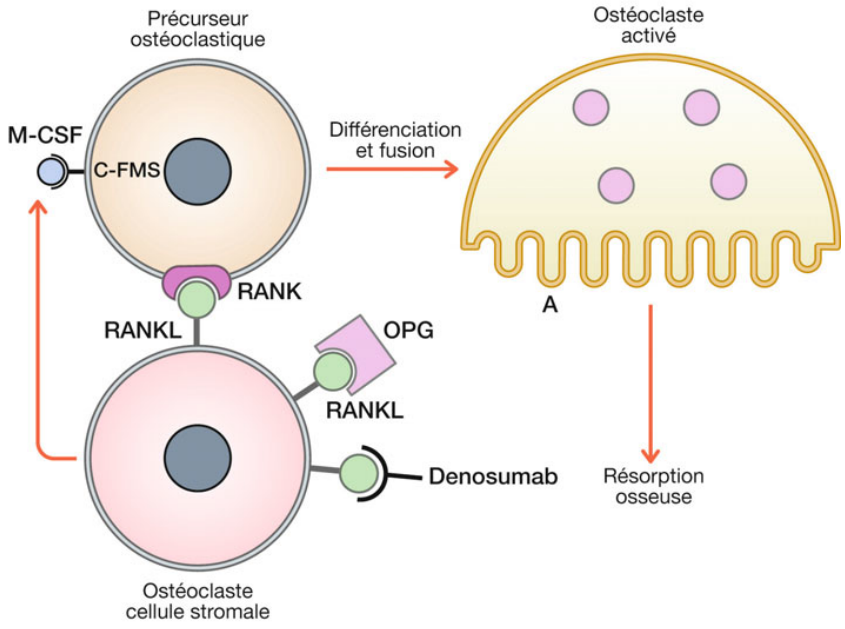

Figure 8 : L'ostéoclastogénèse : régulation, différenciation et activation[20]

1.1.4. Le système vasculaire osseux

La vascularisation osseuse est essentielle pour de nombreux processus, tels que le développement et la croissance du squelette, les phases de remodelage osseux et de cicatrisation osseuse[21].La vascularisation osseuse se fait par les artères et les veines, on retrouve 3 types d'artères :

- Diaphysaires, qui pénètrent par un trou nourricier puis se ramifient dans le canal médullaire. Elles vascularisent la partie interne de l'os compact et la moelle jusqu'à la métaphyse.

- Métaphysaires et épiphysaires, situées à l'extrémité des os, elles irriguent l'os spongieux.

- Périostées, qui sont des ramifications des artère précédemment mentionnées et vascularisent la partie externe de l'os compact.

Les veines doublent les artères et drainent l'os en parcourant sa partie spongieuse.

Comme pour tout autre organe, le système sanguin apporte oxygène et nutriments. Il est également hautement impliqué dans les activités métaboliques et hématopoïétiques du tissu osseux. La vascularisation est étroitement liée aux activités de remodelage et de réparation osseuse de par la présence des capillaires artériels dans les lacunes de résorption et de sinus veineux associés à la néoformation osseuse[22]. Les péricytes (ou cellules de Rouget) qui entourent les capillaires ont la particularité d'exprimer les marqueurs spécifiques des MSCs. Ce sont des cellules multipotentes avec un potentiel de différenciation en cellules adipocytaires, ostéoblastiques et phagocytaires lorsqu'elles sont dissociées de leur tissu d'origine. Les péricytes sont donc impliqués dans les processus de régénération osseuse. En effets ils sont capables de synthétiser du collagène, des protéoglycanes et l'ostéonectine par l'intégration du phosphate de calcium afin de participer à la production de MEC osseuse[23]. Lors d'un événement provocant une stimulation des processus de formation osseuse, on observe une augmentation du nombre de vaisseaux, du débit sanguin et la formation de néo vaisseaux. La néovascularisation lors du remodelage ou de l'ostéogénèse va permettre le dialogue intercellulaire entre les cellules du tissu osseux et les cellules du tissu vasculaire. Cette communication repose, d'une part, sur la sécrétion de facteurs de croissance. En effet les cellules endothéliales et ostéoblastiques communiquent par l'intermédiaires des facteurs sécrétés, tels que le facteur de croissance de l'endothélium vasculaire (VEGF), facteur angiogènique qui à l'origine de cette communication, mais également d'autres facteurs de croissance comme les BMPs. Ces facteurs de

croissance agissent par effet autocrine ou paracrine. D'autre part, la communication entre les cellules ostéoblastiques et endothéliales reposent sur la création de jonctions cellulaires. Ces jonctions peuvent être communicantes par l'action des connexines[24], adhérentes par l'action des cadhérines[25] ou interagir avec la MEC par l'action des intégrines[26].

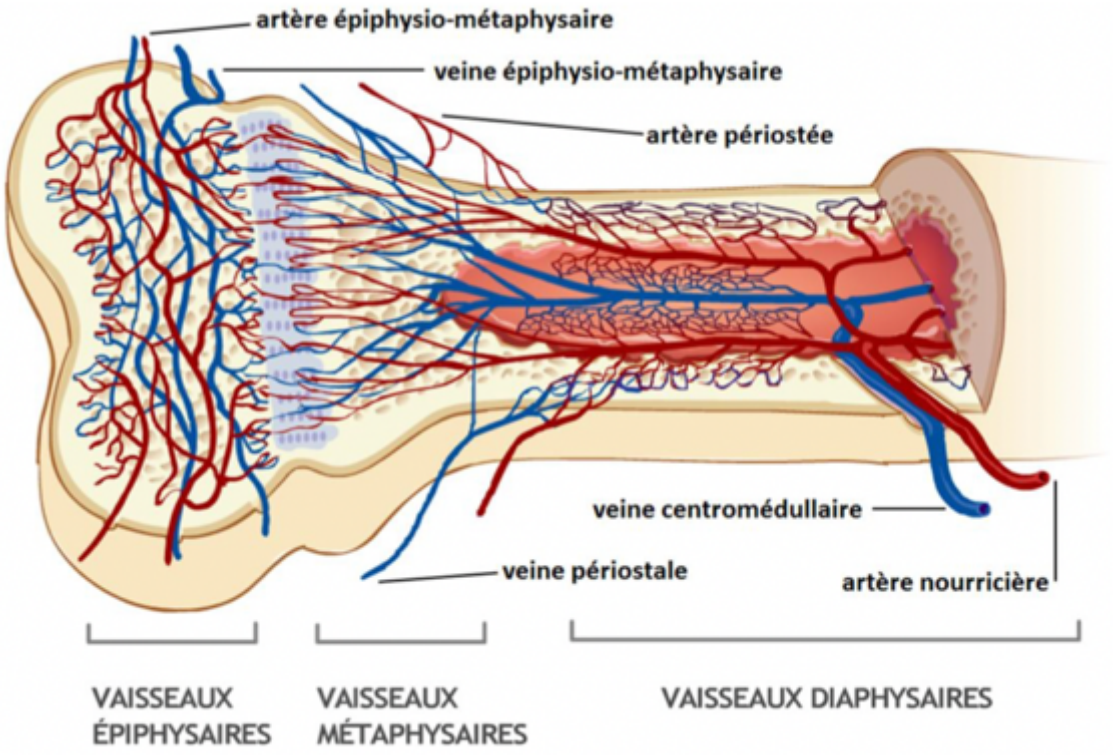

Figure 9 : Schéma de représentation de la vascularisation du tissu osseux[27]

1.1.5. Le remodelage osseux

La croissance osseuse, le modelage et le remodelage sont des processus qui garantissent l'adaptation à la croissance d'un individu, l'intégrité structurale et la régulation de l'homéostasie minérale. La croissance osseuse (longitudinale et radiale) survient principalement pendant l'enfance et l'adolescence. Le modelage consiste en la transformation progressive de l'organisation osseuse en réponse aux forces mécaniques appliquées. Le remodelage osseux est le processus continu par lequel le tissu osseux est renouvelé pour maintenir son intégrité structurale, sa résistance mécanique et pour contrôler l'homéostasie minérale.

Chez l'adulte, le taux de renouvellement du tissu osseux est en moyenne d'environ 10% par an. Ce processus est étroitement régulé par l'action orchestrée de plusieurs types

de cellules disposés dans des compartiments temporaires de remodelage osseux connus sous le nom d'unités multicellulaires de base ou d'unités métaboliques osseuses (BMU). Lorsque le signal de remodelage est initié par les ostéocytes (mécano-senseurs), la phase de résorption osseuse réalisée par les ostéoclastes commence et dure environ 2 à 4 semaines. Ensuite, les ostéoclastes subissent une apoptose, et sont substitués par des cellules mononucléaires d'un phénotype encore peu clair (cellules d'inversion) qui terminent la phase de résorption et préparent la surface pour le dépôt d'une nouvelle matrice[28]. La lacune de résorption est ensuite occupée par les ostéocytes libérés depuis la matrice résorbée et par les pré-ostéoblastes différenciés et recrutés pour la phase de dépôt de matrice (4 à 6 mois). Ce processus est régulé par des facteurs dérivés de la matrice tels que TGF-β, IGF (Insulin-like Growth Factor) et FGF (Fibroblast Growth Factor) et par l'effet des sollicitations mécaniques. Les ostéoblastes déposent alors le tissus ostéoïde qui sera ensuite minéralisé[28,29].

Il existe un équilibre entre ces deux processus d'apposition et de résorption du tissu osseux, le déséquilibre entre ces deux processus conduit à des processus pathologiques telles que l'ostéoporose caractérisée par une perte de densité osseuse (résorption >apposition) et l'ostéopétrose caractérisée par une augmentation de cette densité (apposition>résorption)[30].

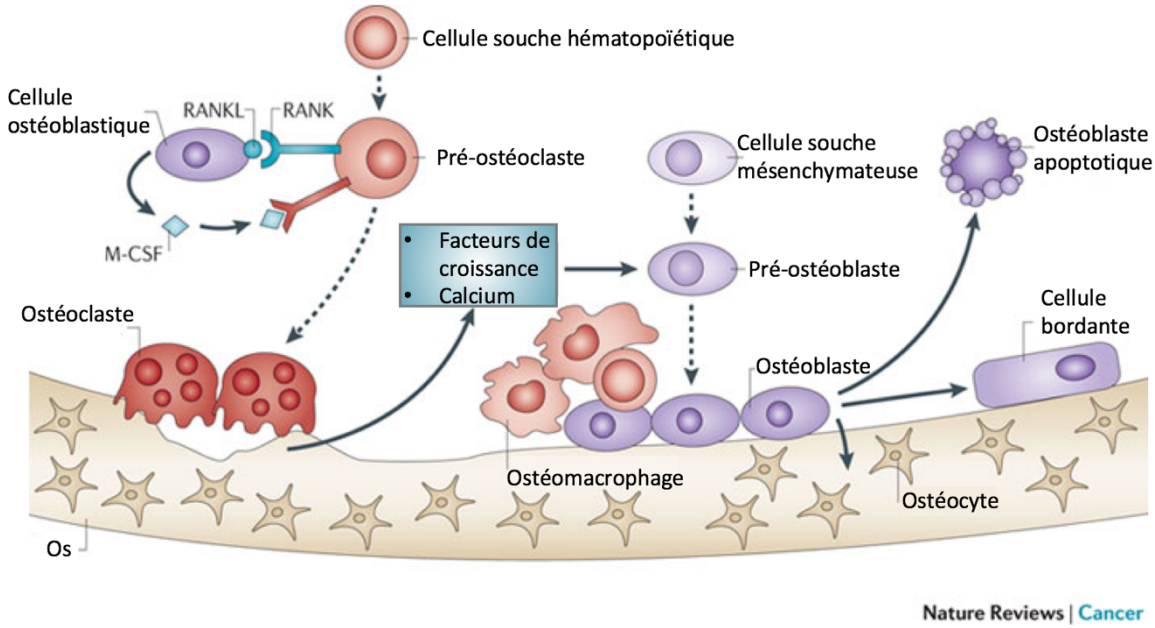

Figure 10 : Représentation schématique du processus de remodelage osseux naturel et cascades de différenciation cellulaire (adapté)[31]

1.1.6. La cicatrisation osseuse

Le tissu osseux a une capacité intrinsèque à se réparer. Les processus de réparation osseuse ne sont pas entièrement compris, mais leur compréhension est la clé de la conception et du développement de nouvelles stratégies efficaces pour le traitement des lésions osseuses de taille critique (trop importante pour une réparation *ad-integrum*). Lors d'une fracture, l'intégrité du squelette est localement perdue et le réseau vasculaire osseux est perturbé, entraînant une altération du flux de nutriments et d'oxygène, affectant la structure de la moelle. Le processus de régénération tissulaire commence ensuite avec trois phases principales: la phase inflammatoire, la phase de prolifération (phase d'union) et la phase de remodelage osseux[32].

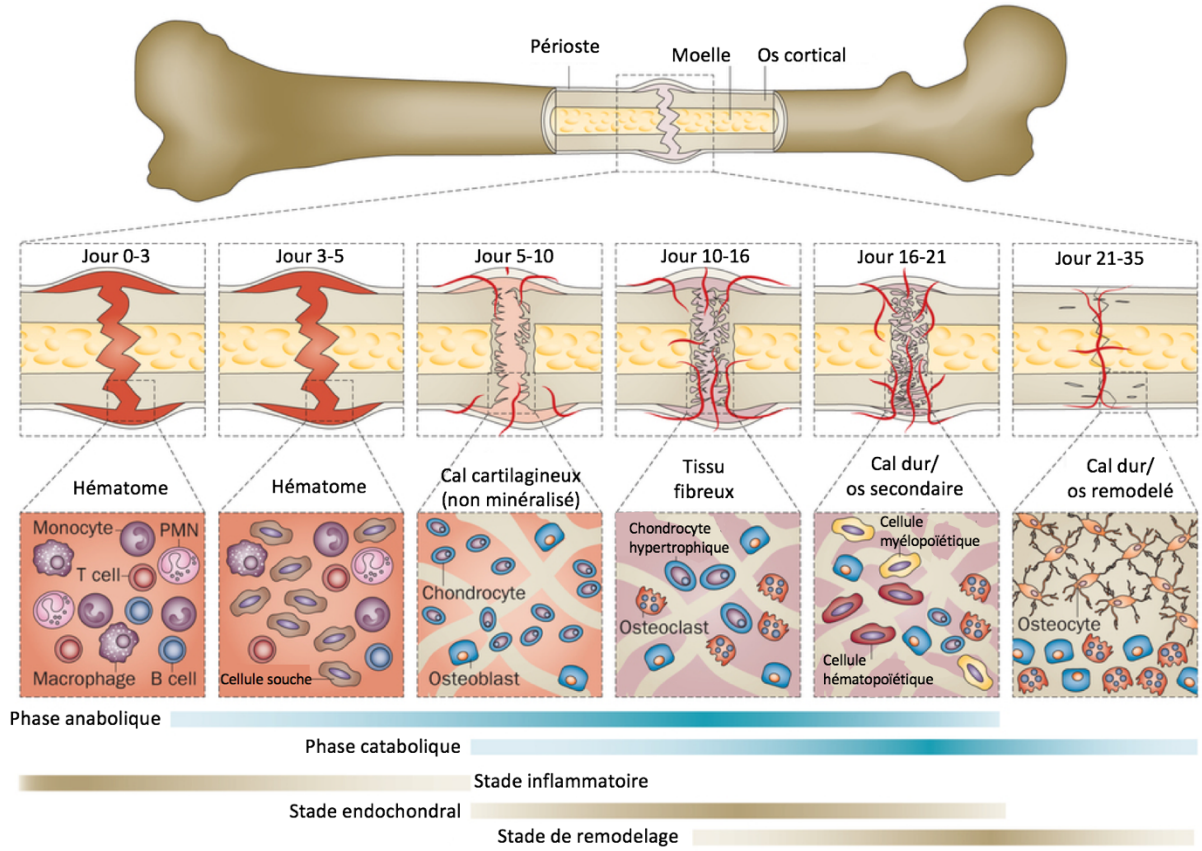

Figure 11 Processus de cicatrisation osseuse (adaptée)[33]

1.1.6.1. La phase inflammatoire

Dans la phase d'inflammation, un caillot de sang (hématome) est formé localement et des facteurs de croissance (IGF-I, facteur de croissance dérivé des plaquettes (PDGF)) et des cytokines sont libérés pour recruter et réguler l'action des macrophages et des précurseurs d'ostéoblastes et de chondroblastes. Ensuite, les cellules immunitaires recrutées sécrètent des molécules de signalisation (FGF, TNF-α), des VEGF, des TGF-β, des interleukine-1 et -6 (IL-1, IL-6). Ces molécules et facteurs stimulent la synthèse de MEC ainsi que l'angiogenèse et permettent le recrutement d'autres cellules inflammatoires ainsi que des précurseurs de cellules mésenchymateuses (provenant principalement du périoste). Celles-ci prolifèrent et se différencient en cellules de lignées ostéogéniques et chondrogéniques formant finalement un tissu de granulation transitoire (cal de granulation)[34].

1.1.6.2. La phase de prolifération (ou phase d'union)

Dans la phase de prolifération (ou phase d'union), l'ostéogénèse est produite par l'un des deux processus d'ossification suivants : l'ossification endochondrale ou l'ossification membranaire. Dans l'ossification endochondrale, les chondroblastes déposent un calcartilagineux qui relie et stabilise le site de fracture. Ce cal est ensuite calcifié, vascularisé et progressivement substitué par de l'os tissé (action des ostéoblastes). Ce

tissu provisoire est mécaniquement faible et est caractérisé par une organisation très aléatoire des fibres de collagène. Lors de l'ossification membranaire, les os compacts et trabéculaires sont directement fabriqués par des ostéoblastes (sans phase intermédiaire de dépôt cartilagineux). Ce processus est principalement limité aux régions sous-periostées (fracture avec déplacement peu marqué des fragments, sans ou avec légère rupture du périoste)[35] où le réseau capillaire est resté fonctionnel[34].

1.1.6.3. La phase de remodelage

Enfin, dans la phase de remodelage, le cal provisoire est transformé en un nouveau tissu osseux avec une structure lamellaire et une cavité médullaire interne restaurant ainsi les propriétés biomécaniques de l'os[35]. Comme lors du processus de remodelage osseux des os intacts, la phase de remodelage qui intervient lors de la cicatrisation osseuse est basée sur l'action des BMU. Elle consiste en une combinaison de résorption opérée par les ostéoclastes et de dépôt de matrice osseuse réalisé par les ostéoblastes. Cette phase peut prendre des mois avant d'obtenir l'os entièrement réparé, identique à l'os natif[35].

Dans le cas d'importantes fractures ou pathologies causant la perte de grandes quantités osseuses, le tissu ne peut cicatriser naturellement. Il sera alors nécessaire d'avoir recours à des biomatériaux de remplacement ou de comblement et/ou à la réalisation de greffes pour restaurer les propriétés mécaniques et/ou biologique de l'organe lésé.

1.1.7. Les principaux types de lésions osseuses et leurs causes pathologiques

L'objectif de ce chapitre n'étant pas de répertorier la totalité des lésions et pathologies, cette section présente les principales affections du tissu osseux sans toutefois être exhaustive.

1.1.7.1. Traumatismes

Comme il a été décrit précédemment, le tissu osseux présente des propriétés structurales qui lui confèrent une résistance aux contraintes mécaniques de par son élasticité. Néanmoins, quand les contraintes dépassent les capacités d'amortissement de l'os celui-ci peut se fracturer. Les traitements de fractures diffèrent principalement selon la gravité de la fracture (avec ou sans déplacement, fermée ou ouverte, stable ou instable) allant de la simple contention à l'intervention chirurgicale avec pose de moyens internes et/ou externes.

1.1.7.2. Fractures de stress

Les fractures de fatigue ou de « stress » sont principalement rencontrées chez les sujets sportifs, sur un os sain où un stress répété ne permet pas aux ostéoblastes de remodeler l'os endommagé entrainant ainsi un déséquilibre entre résorption et formation osseuse. Bien que dans la majorité des cas le repos et la diminution de la mise en charge de l'articulation suffisent à la guérison une chirurgie peut être nécessaire pour les fractures de stress graves[36].

1.1.7.3. Origine Infectieuse

L'ostéite est une inflammation du tissu osseux, aiguë ou chronique, généralement causée par infection bactérienne (mais pouvant également être d'origine traumatique, tumorale, iatrogènes)[37]. Le traitement repose sur l'association d'un geste chirurgical accompagné d'antibiothérapie. Le geste chirurgical vise à extraire le tissu osseux infecté alors que les antibiotiques éradiquent les germes pathogènes. Lorsque la perte osseuse est importante, une greffe est réalisée pour compenser la perte.

1.1.7.4. Tumeurs

Les métastases sont les tumeurs osseuses les plus fréquentes. Elles correspondent à la dissémination de cellules tumorales à distance de la tumeur initiale qui vont coloniser le tissu osseux[38]. Les myélomes et d'autres formes de cancers (appelés tumeurs ostéolytiques) peuvent entrainer une ostéolyse qui est une destruction du tissu osseux. Généralement en association avec d'autres traitements thérapeutiques (chimiothérapie, radiothérapie…) un geste chirurgical sera nécessaire. Il s'agit de réaliser une résection osseuse afin d'enlever la tumeur en totalité avec des marges appropriées évitant ainsi une récidive locale[39].

1.1.7.5. Troubles du métabolisme osseux

L'ostéoporose est une maladie qui se caractérise par une fragilité importante du tissu osseux ce qui augmente le risque de fracture[40]. Cette pathologie est la conséquence du déséquilibre entre les deux processus du remodelage osseux où les mécanisme de résorption prédominent sur l'apposition de nouvelle matrice osseuse[30]. C'est la cause principale des fractures osseuses chez les personnes âgées, elles surviennent principalement à la hanche, aux vertèbres ou à l'avant bras[41].

L'ostéomalacie se caractérise par une décalcification osseuse causée par une insuffisance de minéralisation (manque d'ions calcium et phosphate). A un stade avancé, cette pathologie entraine l'apparition de microfissures généralement localisées sur les vertèbres, les hanches et le bassin ainsi que des fractures osseuses spontanées.

1.1.7.6. Autres types de pertes osseuses physiologiques

Les contraintes mécaniques stimulent l'ostéo-formation. En leur absence comme lors de la perte de dents, une résorption osseuse est observée. En effet, à la suite de la perte d'une dent ou d'une extraction une perte d'un tiers du volume de la paroi vestibulaire osseuse a été identifiée[42]. Afin d'offrir un capital osseux suffisant et une zone d'ancrage solide pour la pose d'un implant le chirurgien aura recours à des techniques de comblement osseux : l'augmentation par greffe osseuse ou la régénération osseuse guidée[43].

1.1.8. Stratégies cliniques de thérapies et comblements osseux

Il a été vu précédemment que le tissu osseux possède une capacité de cicatrisation naturelle dans le cas de lésion de faible volume. Lors d'un traumatisme, d'une pathologie ou d'un acte chirurgical où la perte de substance osseuse est trop importante la mise en place d'une stratégie de comblement ou d'aide à la cicatrisation osseuse est nécessaire. Les stratégies cliniques appliquées au tissu osseux sont fonction du type de lésion, de leur origine, du site concerné et de leur gravité. Du fait des propriétés mécaniques des hydrogels, nous traiterons dans les parties suivantes des stratégies cliniques employées en site osseux où les mises en charges sont faibles ou en complément de matériaux d'union ou de stabilisation mécanique.

1.1.8.1. Les autogreffes

L'autogreffe est considérée comme le traitement de référence en application clinique[44]. Cette technique est définie comme la transplantation d'un tissu d'un site à un autre chez un même individu. Il s'agit de prélever chez le patient son propre tissu osseux sur un site donneur puis de le placer sur le site receveur. Le prélèvement et la greffe sont réalisés lors de la même intervention chirurgicale. Il sera généralement effectué sur la crête iliaque, plus rarement le tibia ou le sternum[45].Les greffes osseuses réalisées à partir d'autogreffes offrent un taux de succès élevé car elles sont essentiellement constituées de tissus vivants composés de cellules intactes et s'affranchissent du risque de réaction immunitaire. De plus les prélèvements d'os cortical contenant le pédicule vasculaire permettent d'accélérer le processus de consolidation de la greffe[46]. Cette technique présente cependant certaines limites. Elles sont dues à la quantité limitée de tissu osseux pouvant être prélevé chez un patient ainsi qu'à la morbidité inhérente au second site chirurgical. De plus cette technique sera à proscrire chez les patients présentant des pathologies osseuses comme par exemple l'ostéoporose.

1.1.8.2. Les allogreffes

L'allogreffe peut être une alternative à l'autogreffe dans certains cas, par exemple lors de pertes très importantes de substances osseuses ou chez les patients âgés présentant un faible capital osseux[47]. Comme pour l'autogreffe, les allogreffes osseuses proviennent de tissus humains à la différence qu'ils sont issus d'un autre individu (receveur et donneur différents). La source du greffon sera le prélèvement sur des cadavres qui ont fait don de leurs organes ou plus généralement des déchets chirurgicaux. En effet les banques de tissus osseux peuvent fournir des allogreffes provenant de résections osseuses réalisées lors d'arthroplasties totales de hanches dans le cas où le patient répond à un certain nombre de critères stricts[48].Il existe trois types d'allogreffes osseuses disponibles : le tissu osseux frais ou congelé, l'allogreffe osseuse lyophilisée, l'allogreffe lyophilisée déminéralisée[49]. Malgré la bonne disponibilité des allogreffes, celles-ci présentent des limites. Les allogreffes fraîches présentent une faible ostéogénicité et un potentiel immunogène très élevé. Celles traitées par congélation, lyphylisation ou irradiation présentent un risque de contamination bactérienne et virale (hépatite, HIV). D'autre part leur résorption est parfois trop lente. Bien qu'elles proviennent essentiellement de déchets chirurgicaux leur coût reste très élevé (sérologies, quarantaine et stockage à -80°C). De plus, les méthodes de préparation et conservations peuvent altérer les propriétés biomécaniques des allogreffes[50,51].

1.1.8.3. Les xénogreffes

Les xénogreffes sont des greffes osseuses provenant d'une espèce différente de celle du receveur, telles que les xénogreffes d'origine bovines utilisées comme matrice calcifiée implantée chez l'Homme[49]. Il existe différentes xénogreffes commercialisées d'origine bovine (Pyrost®, Cerabone®, BioOss®, Ostéovit®, Laddec/Lubboc®), équine (Equimatrix®) ou porcine (Osteobiol®). Différents traitements sont nécessaires à la préparation de ces greffes osseuses : pyrolysation, décalcification, déprotéination, délipidation[51]. Ces traitements vont altérer les protéines matricielles et/ou modifier l'état de surface ce qui aura un impact néfaste sur les mécanismes d'adhérence, de prolifération et de différenciation cellulaire[52].

1.2. Stratégies alternatives aux techniques actuelles : L'ingénierie tissulaire

Compte tenu des limitations que présentent les techniques précédemment mentionnées telles que décrites dans le tableau suivant, les chercheurs travaillent sur des alternatives prometteuses comme l'ingénierie tissulaire

Tableau 1 : Stratégies cliniques actuelles et principales limitations

Limitations	Autogreffe	Allogreffe	Xénogreffe
Adaptabilité du greffon à la géométrie de la lésion osseuse	+/-	+/-	+/-
Caractère de l'intervention chirurgicale[53,54]	Très invasif	Invasif	Invasif
Morbidité du site donneur[55,56]	Risque très important	-	-
Risque de transmission de maladies[54,57]	Faible	Élevé	Élevé
Propriétés mécaniques et biologiques[58–60]	Bonnes	Moyennes	Faibles
Coût économique	Faible	Très important	Important
Disponibilité	Faible	Bonne	Très bone

1.2.1. L'ingénierie tissulaire

L'ingénierie tissulaire représente un domaine de recherche où des progrès encourageants ont été réalisés ces dernières décennies. Les techniques d'ingénierie tissulaire reposent sur le principe de la restauration de la fonction anatomique / physiologique / biomécanique ou du remplacement des tissus endommagés ou pathologiques grâce à l'application des principes de biologie et d'ingénierie[61].

La première application mondiale du génie tissulaire est une transplantation d'épiderme sur un grand brûlé réalisée par l'équipe de Howard Green, à Boston en 1983[62,63]. Le traitement consistait à prélever une petite superficie de peau saine, à la cultiver *in vitro* et à la greffer par la suite sur le même patient. Depuis plusieurs années, les grands brûlés peuvent être soignés de cette façon, mais la technique doit être améliorée afin de fabriquer un tissu qui réunirait toutes les caractéristiques de la peau d'origine. En plus du domaine cutané, les chercheurs veulent étendre cette thérapie par ingénierie tissulaire à d'autres domaines :

- Orthopédique, avec le remplacement ou la réparation du cartilage, du tissu osseux ou encore des ligaments ;
- Vasculaire, avec la reproduction des trois couches distinctes des vaisseaux sanguins ;
- Pneumologique, avec la reproduction des structures bronchiques à des fins expérimentales ;

- Ophtalmologique, avec la reconstruction de cornée humaine ;
- La Construction de néo-organes comme le cœur, le foie ou encore la vessie

La finalité de l'ingénierie tissulaire est essentiellement clinique, mais également expérimentale, intéressant les chercheurs qui travaillent dans les domaines de la physiologie, de la pathologie et de la pharmacologie. Les organes créés grâce à cette technique sont proches des tissus d'origine et permettent ainsi de tester des produits sans risque pour les humains[64].

1.2.2. L'ingénierie tissulaire osseuse

Cette stratégie appliquée au tissu osseux est appelée l'ingénierie tissulaire osseuse. En réponse au besoin urgent d'offrir de nouveaux traitements à la reconstruction des lésions osseuses, l'ingénierie tissulaire osseuse propose une approche prometteuse pour régénérer efficacement l'os et contourner les limitations associées aux traitements conventionnels[65]. Les principales applications ayant illustré son utilisation comprennent le comblement de pertes osseuses, le comblement de lacunes dans la fusion spinale et la stabilisation de fractures vertébrales par compression[66–68].

Les stratégies pour l'ingénierie tissulaire osseuse ont continuellement évolué depuis les 30 dernières années et l'apparition du concept d'«ingénierie tissulaire» en 1987[69]. Les avancées faites depuis les dernières décennies dans les domaines des biomatériaux, des cellules souches, de la biologie du développement et leur convergence vers les sciences du génie tissulaire ont permis de grandes avancées.

L'ingénierie tissulaire osseuse exige un travail collaboratif des scientifiques, des ingénieurs et des chirurgiens pour atteindre cet objectif ultime consistant à créer des greffes osseuses qui permettent de s'affranchir des limites des traitements actuels. Les techniques d'ingénierie osseuses appliquées au tissu osseux font appel à plusieurs acteurs : (1) un échafaudage biocompatible qui imite les niches de la matrice extracellulaire des os naturels, (2) des cellules ostéogéniques qui synthétisent et déposent la matrice osseuse, (3) des signaux morphogéniques qui participent au recrutement et à la différenciation cellulaire vers le phénotypesouhaitable[70]. Les stratégies d'ingénierie tissulaire pourront être divisées en trois catégories : la combinaison de (1) et (2), la combinaison de (1) et (3), la combinaison de (1), (2) et (3). Ces stratégies sont explicitées ci-après.

1.2.2.1. Stratégie échafaudage-cellules

Cette stratégie repose sur l'apport de cellules exogènes ensemencées au sein d'un échafaudage qui sera implanté sur le site lésé. L'objectif est d'apporter dans une zone anatomique définie des cellules pluripotentes ou prédifférenciées en cellules ostéogéniques. Après avoir été prélevées, isolées et amplifiées les cellules sont ensemencées au sein de l'échafaudage. Cette construction pourra être implantée immédiatement après sa formation ou après mise en culture, généralement dans un bioréacteur, afin de pré-différencier les cellules présentes au sein du matériau d'échafaudage en cellules de la lignée ostéogénique[71]. Les matériaux sélectionnés afin de concevoir les échafaudages devront par leurs propriétés permettre l'adhésion, la survie et la différenciation cellulaire. Lorsque ce complexe échafaudage-cellule est implanté, les cellules participeront aux processus de régénération osseuse, notamment en synthétisant une nouvelle matrice osseuse alors que le matériau échafaudage se dégradera pour laisser place au tissu osseux nouvellement formé[72].

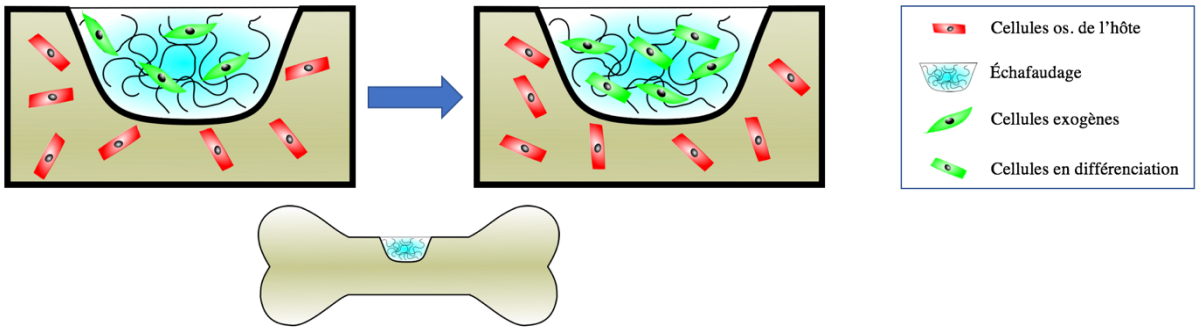

Figure 12 : Stratégie Echafaudage - Cellules souches exogènes

1.2.2.2. Stratégie de libération de molécules par l'échafaudage

Cette seconde stratégie a pour objectif d'apporter à la zone lésée des molécules de signalisation qui stimulent les processus de régénération osseuse par les cellules de l'hôte présentes au voisinage de la lésion. La libération de ces molécules (facteur de croissance par exemple) au sein du site lésé doit être parfaitement contrôlée et optimisée afin de promouvoir la formation d'un tissu osseux sain et éviter les complications comme la formation osseuse ectopique.

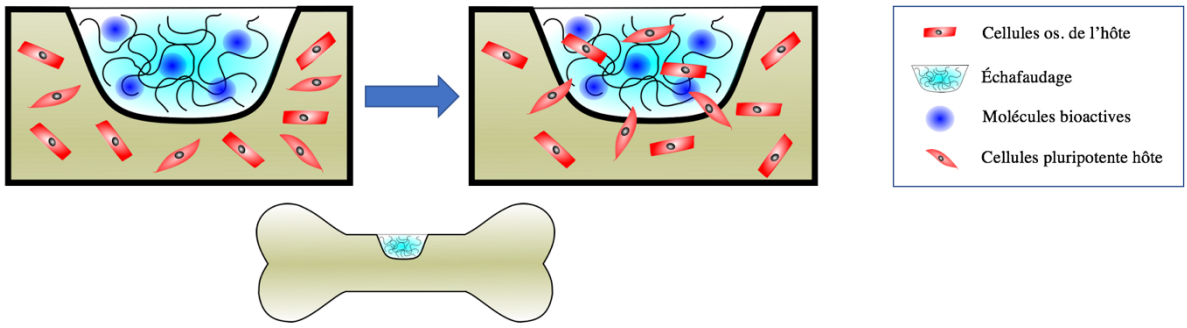

Figure 13 : Stratégie Échafaudage - Molécules bioactives

Dans ce contexte, la stratégie consiste à lier chimiquement ou à encapsuler physiquement les molécules au sein du matériau d'échafaudage. Ainsi les molécules libérées par l'échafaudage exerceront leur rôle au sein du site lésé en favorisant le recrutement et la différenciation cellulaire pour la formation d'une nouvelle matrice osseuse[73].

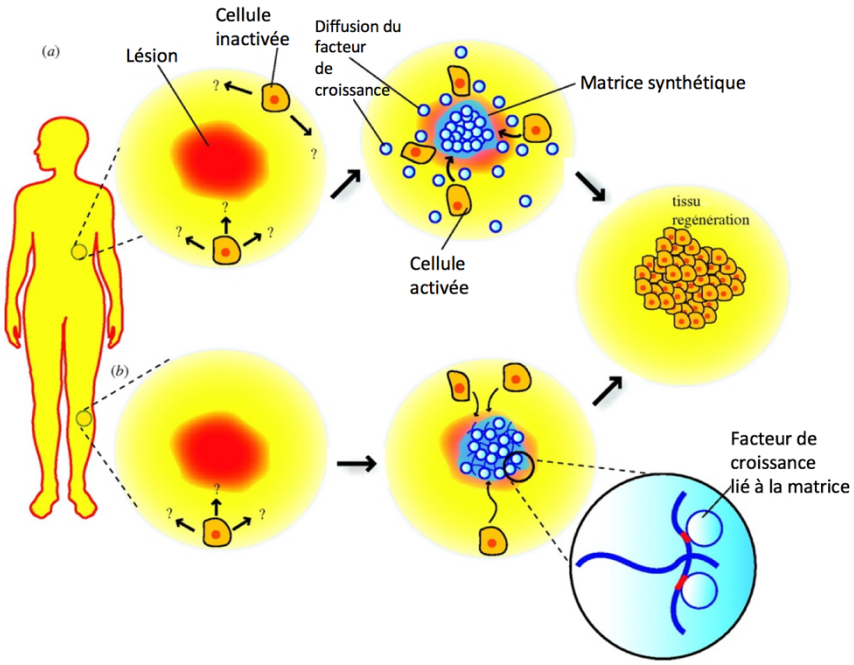

Figure 14 : Stratégies de liaison chimique ou d'encapsulation physique des molécules bioactives au sein de l'échafaudage (adaptée)[73]

1.2.2.3. Stratégie échafaudage-cellule-signaux morphogéniques

La troisième stratégie consiste à associer les trois composantes mentionnées précédemment. L'échafaudage devra donc présenter à la fois des cellules exogènes et des molécules de signalisation comme par exemples des facteurs de croissance. Les molécules libérées pourront stimuler les cellules exogènes ensemencées au sein de l'échafaudage et les cellules de l'hôte, de façon à coordonner le processus de régénération osseuse.

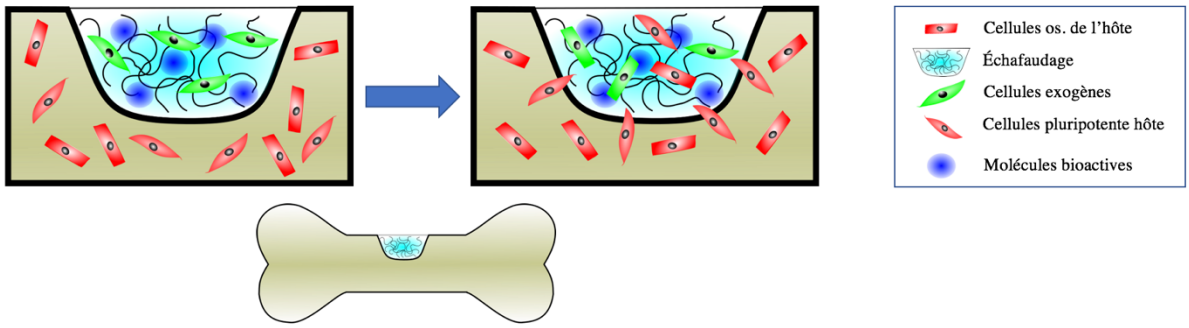

Figure 15 : Stratégie échafaudage-cellule-signaux morphogéniques

Les prochaines parties (La source cellulaire en ingénierie tissulaire osseuse ; La matrice d'échafaudage en ingénierie tissulaire osseuse ; Le rôle des facteurs de croissance en ingénierie tissulaire osseuse) seront à but introductif puisque ces thèmes sont plus largement abordés dans le chapitre 3 « Cellularizing hydrogel-based scaffolds to repair bone tissue: How to create a physiologically-relevant micro-environment? »

1.2.2.4. La source cellulaire en ingénierie tissulaire osseuse

L'ingénierie tissulaire comme la médecine régénératrice sont des sciences multidisciplinaires qui ont évolué en parallèle avec les récentes avancées biotechnologiques.

Malgré ces progrès, la disponibilité de cellules souches reste un défi pour les scientifiques et les cliniciens. Les cellules sont responsables de la synthèse et de la stabilisation à long terme de la MEC, cette dernière étant importante pour générer un nouveau tissu[74]. L'interaction matrice/cellule, l'adhésion et la prolifération des cellules ainsi que la production de la MEC sont des critères importants pour réussir à former un tissu[75].

Idéalement, une cellule souche pour des applications en ingénierie tissulaire doit répondre aux critères suivants :

- Être disponible en quantité suffisante
- Être isolée à partir d'une procédure peu invasive
- Être différenciée de manière contrôlée et reproductible
- Être transplantée en toute sécurité et efficacement au sein de la lésion
- Être produite dans le respect des directives de bonnes pratiques de laboratoire

1.2.2.5. La matrice d'échafaudage à base d'hydrogel en ingénierie tissulaire osseuse

Parmi les biomatériaux les plus utilisés en ingénierie du tissu osseux, seront cités les matériaux macroporeux à base de phosphate de calcium, les polymères synthétiques, naturels et/ou composites [76] qui donnent des résultats très prometteurs sur la différenciation des cellules souches, surtout dans des conditions dynamiques de culture[77].

La reconstruction de lésions osseuses est un important enjeu en clinique, en particulier dans la région cranio-faciale à la suite de malformations congénitales, de traumatismes, d'accidents ou de maladies. Il serait idéal d'accéder à ces sites lésés en utilisant des techniques mini-invasives qui laisseraient des cicatrices minimes et réduiraient au minimum l'inconfort du patient. C'est dans ce contexte que l'ingénierie tissulaire utilisant les hydrogels peut offrir une approche intéressante pour l'administration peu invasive, l'intégration avec le tissu hôte et la régénération osseuse[78].

Les hydrogels ont déjà trouvé de nombreuses applications pour les systèmes à libération contrôlée de médicaments et facteurs de croissance[79,80] et sont généralement biocompatibles, car ils ont une similarité structurale avec les macromolécules du corps humain[81]. Ils ont également été utilisés pour des applications en ingénierie tissulaire, car ils sont un bon support à l'injection de cellules ou de molécules bioactives et leur administration est peu invasive[82,83]. Cette forme d'administration d'un produit d'ingénierie tissulaire peut diminuer la douleur ressentie par le patient, le coût et la complexité d'une intervention chirurgicale de routine.

Une grande variété d'hydrogel, matériaux fortement hydratés (teneur en eau ≥ 30%)[84] peuvent être employés comme supports pour l'ingénierie tissulaire. Ils peuvent être

d'origine synthétique ou naturelle, leurs propriétés et leur intégrité dépendront, en plus de leur composition, des interactions chimiques ou physiques qui maintiennent leur structure. Les hydrogels, comme tout matériau destiné à être implanté chez l'Homme doit répondre à un cahier des charges précis et donc présenter plusieurs propriétés[85] :

- Biocompatibilité : est la capacité des matériaux à ne pas interférer, ne pas dégrader, le milieu biologique dans lequel ils sont utilisés[85].

- Non-cytoxicité : La dégradation du matériau ne doit pas conduire à la libération de substances cytotoxiques[86].

Les matériaux destinés à la régénération des tissus osseux doivent répondre à un cahier des charges adaptés aux propriétés de celui-ci. Ainsi il devra également favoriser :

- L'ostéoinduction : le matériau est capable de promouvoir l'ostéogenèse au sein de la matrice implanté, en stimulant les cellules associées vers une différenciation en cellules osseuses (préostéoblastes, ostéoblastes)[87].

- L'ostéoconduction : le matériau doit être apte à servir de support à la formation d'os natif autour de l'implant, de façon à assurer une continuïté structurale et fonctionnelle entre l'implant et l'os natif [87].

- L'ostéogénicité : le matériau à la capacité de contenir des cellules ostéogéniques qui survivront lors de son implantation, l'ostéogenèse sera donc induite par les cellules qu'apporte ou recrute le matériau[88,89].

1.2.2.6. Le rôle des facteurs de croissance en ingénierie tissulaire osseuse

Les facteurs de croissance sont des protéines sécrétées par les cellules et qui fonctionnent comme des molécules de signalisation. Ces substances protéiniques, également appelés premiers messagers, ont un effet autocrine (effet sur la cellule sécrétrice), paracrine (effet dans le voisinage de la cellule sécrétrice) ou endocrine (sécrétion dans le système sanguin) sur une cellule cible donnée. En plus de favoriser la différenciation cellulaire, ils ont un effet direct sur l'adhésion, la prolifération et la migration cellulaire en modulant la synthèse des autres facteurs de croissance et des récepteurs cellulaires[90].Depuis de nombreuses années, une attention toute particulière est portée sur le rôle des protéines morphogéniques osseuses (BMPs) et leur rôle dans la formation osseuse embryologique ainsi que dans les processus de réparation osseuse[90,91]. Les BMPs sont un groupe de glycoprotéines non-collagéniques qui appartiennent à la superfamille des TGF-β. Les BMPs sont

synthétisées localement et exercent leurs effets principalement par des effets autocrines et paracrines. À ce jour plus de 15 BMPs différentes ont été mises en évidence, les plus étudiées sont la BMP-2, la BMP-3 et la BMP-7[92] du fait de leur rôle important dans la régénération osseuse par stimulation de la différenciation des MSCs vers les lignées ostéogéniques.

2. Approche et objectifs

Le défi que doit relever l'ingénierie du tissu osseux est de proposer une alternative pertinente aux solutions cliniques actuelles dont nous avons décrit les limites. Cependant, comme le montrent les sections précédentes, la complexité du tissu osseux requiert la prise en charge de nombreux paramètres dans le développement de nouvelles stratégies de comblement et réparation du tissu osseux. Dans la mise en place de ces stratégies basées sur le génie tissulaire de nombreux défis persistent, notamment dans le développement des matériaux d'échafaudage et des stratégies d'apport cellulaire et de libération de molécules bioactive au sein du site osseux lésé. Comme décrit précédemment, les stratégies de régénération tissulaire sont souvent divisées en 3 catégories. Au cours de nous travaux nos développeront des stratégies basées sur l'apport de cellules souches exogènes puis sur la libération de protéines via des matrices d'hydrogels.

2.1. Les éléments constitutifs des stratégies d'ingénierie tissulaire osseuse

2.1.1. Les cellules souches issues du tissu adipeux (hASCs)

Les cellules souches dérivées du tissu adipeux humain (hASCs) ont la capacité de se différencier dans plusieurs types cellulaires[93] : adipocytes[94], ostéoblastes[95,96], chondroblastes[97,98], myoblastes[99], fibroblastes[100] et neuroblastes[101].

Le tissu adipeux représente une source de cellules autologue idéale pour des stratégies d'ingénierie tissulaire en raison de la bonne accessibilité de ce tissu. En effet, le tissu adipeux est particulièrement facile à obtenir en grandes quantités en utilisant les techniques de liposuccion ou de résection abdominale. Le tissu adipeux représente environ 10 % du poids corporel chez un individu adulte sain et jusqu'à 50% chez des patients obèses.

Les hASCs ont un potentiel prolifératif supérieur aux cellules souches isolées à partir de la moelle osseuse. Un seul gramme de tissu adipeux humain peut fournir plus de 50000 cellules souches adultes pluripotentes suite à leur extraction[102]. Toute personne peut donc disposer de ses propres cellules souches pour une autogreffe réparatrice, exempte de rejet. Et chacun pourrait les proposer, pour une allogreffe, à un patient qui serait porteur d'un défaut génétique interdisant l'autogreffe.

En plus des capacités de reconstruction des tissus mous, les hASCs offrent un potentiel de réparation des tissus durs comme le cartilage ou l'os. Dans des conditions de culture

ostéogéniques, il a été montré que les hASCs peuvent exprimer des gènes et des protéines propres au phénotype ostéoblastique comme la phosphatase alcaline, l'ostéopontine et l'ostéocalcine[103]. La différenciation ostéogénique sera caractérisée par le changement de morphologie cellulaire vers la morphologie ostéoblastiques (cellules cubiques) et le dépôt d'une matrice extracellulaire composée principalement de collagène de type 1 qui sera rapidement minéralisée. Dans les conditions d'étude *in vivo* il a été montré que l'utilisation d'un échafaudage de soutien approprié ensemencé d'hASCs permet la formation de tissu osseux notamment dans des modèles d'ostéoformation ectopique murins[104].

2.1.2. Les hydrogels de collagène

Le collagène est le principal composant conférant aux tissus conjonctifs, tels que les os, les dents, les cartilages, les tendons, les ligaments et les matrices fibreuses de la peau et des vaisseaux sanguins, leur résistance et leur géométrie[105]. Il s'agit de la protéine la plus abondante dans les tissus des mammifères, en effet il représente environ 25 % de la totalité des protéines. De plus, le collagène constitue la majeure partie organique non minéralisée du tissu osseux. Dans certains types de collagène, la molécule entière est une triple hélice alors que dans d'autres types seule une portion est formée d'une triple hélice. Le collagène mature de type I est formé d'environ 1000 acides aminés et possède cette structure en triple hélice[106]. Le collagène est un polymère naturel largement utilisé dans le domaine de l'ingénierie tissulaire en raison de ses excellentes propriétés biologiques[107]. Les applications biomédicales du collagène sont très nombreuses. Il est utilisé en tant que matrice de support pour la culture cellulaire, de matériau pour certain fils de suture[108] ou d'agent hémostatique[109]. Le collagène est également utilisé pour la conception de systèmes à libération contrôlée de médicaments[110] ou de protéines[111]. Les éponges de collagène sont aussi utilisées pour soigner les brûlures et autres blessures[112]. Il s'agit également d'un support étudié pour la régénération des tissus épidermiques, osseux, vasculaires et des valves cardiaques. Peu de cas de complications ont été enregistrés lors de l'utilisation clinique de dispositifs à base de collagène[113].

Le collagène possède de très faibles propriétés antigéniques et immunogéniques[114]. Les éventuelles réponses immunitaires pouvant survenir lors de l'utilisation de produits contenant du collagène sont essentiellement dues à des composés présents autres que le collagène, tel que des impuretés, ou des traces d'agents de réticulation non éliminés[114].

Selon un protocole établi et publié par le Professeur Mantovani et son équipe de recherche, le collagène de type I est extrait de tendons de queues de rats et stocké selon un protocole élaboré au sein du laboratoire[115]. Les fibres de collagène ainsi obtenues sont solubilisées dans de l'acide acétique. Cette solution est congelée puis lyophilisée afin d'obtenir des éponges de collagène qui peuvent être conservées à -80 °C. Afin de réaliser nos expériences, les éponges sont dissoutes dans de l'acide acétique de sorte à obtenir une solution homogène et légèrement visqueuse de collagène à une concentration finale de 4 g/L.

Avec cette solution, un gel de collagène peut ensuite être réalisé, avec ou sans cellule. La gélification du collagène s'effectue à un pH physiologique. Le protocole utilisé amène progressivement la solution de collagène à un pH neutre par l'ajout de tampon et de milieu de culture cellulaire complet qui apporte les nutriments nécessaires à la viabilité des cellules ensemencées. Il constitue alors un environnement idéal pour la croissance des cellules souches issues du tissu adipeux[116].

Le collagène présente donc des avantages majeurs : il est biodégradable, biocompatible, non immunogène et facilement disponible[117]. Il est également choisi en tant que support pour les cellules souches pour la régénération tissulaire de par ses excellentes propriétés biologiques. Le collagène favorise l'adhésion et la prolifération des cellules, par interaction entre le site RGD de la protéine et les intégrines des membranes cellulaires[118,119]. Cependant les hydrogels de collagène souffrent de leurs propriétés mécaniques faibles, qui entrainent notamment sa compaction (perte de volume) lorsqu'il est support à la croissance des cellules souches issues du tissu adipeux (hASCs). Il semble donc pertinent d'associer les hydrogels de collagène a un hydrogel offrant des propriétés mécaniques limitant la perte de volume et la dégradation trop rapide du matériau lors de son implantation.

2.1.3. L'hydrogel de GNF (Glycosyl Nucléoside Fluoré)

Le Glycosil-Nucléoside-Fluoré (GNF), amphiphile, est une nouvelle classe de molécules gélatrices de faible poids moléculaire possédant un sucre, un nucléoside et des lipides liés de façon covalente par des ponts triazoles. Cette famille est composée de deux catégories principales : les Glycosyl-Nucléo-Lipides (GNLs) qui possèdent une ou deux chaines lipidiques[120] et le GNF à chaines fluorocarbonées, fortement hydrophobes[121]. Le GNF utilisé au cours de nos travaux est constitué de trois groupements : une chaine fluorocarbonée, un groupement thymidine central et un fragment d'hydrate de carbone, la chaine fluorée et l'hydrate de carbone sont tous deux liés à la thymidine centrale.

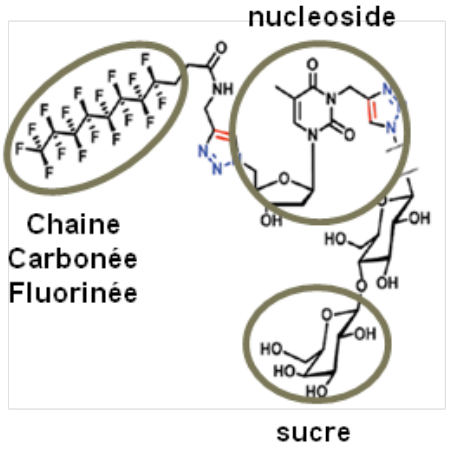

Figure 16 : formule développée du GNF. La chaine fluorocarbonée et le sucre sont liés à une thymidine par des groupements propargyle d'après[121]

Après avoir été solubilisés dans une solution tampon aqueuse à haute température (65 °C) ces composés s'auto-assemblent en structures supramoléculaires hautement organisées formant des hydrogels à la température physiologique. Une augmentation de la température induit une fusion du gel, alors qu'un retour à température physiologique entrainera une nouvelle gélification, on parlera donc d'hydrogel réversible, thermosensible.

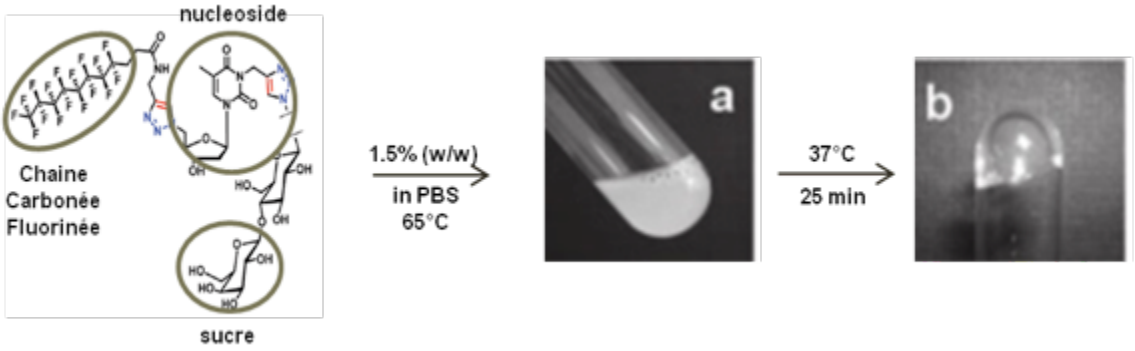

Figure 17 : Transition du GNF de l'état liquide (a) à l'état de gel (b)

De précédentes études ont décrit certaines propriétés de ce nouvel hydrogel. Ce gel se forme en 25 minutes par auto assemblage des monomères lorsque la température diminue, aucun agent de réticulation chimique n'est donc nécessaire. Cela limite le risque de toxicité dû à l'utilisation de molécules potentiellement nocives. La gélification se produit lentement, ce qui permet le mélange des cellules à 37°C avant que le gel ne soit formé.

Ce mécanisme de gélification propose également la possibilité d'injecter la solution GNF à l'état visqueux et de former un gel *in situ*. À la suite de son implantation, une réaction inflammatoire chronique très modérée a été observée ainsi que la vascularisation et la

colonisation de l'hydrogel par les cellules de l'hôte ce qui suggère une bonne intégration à l'environnement d'implantation. Les hydrogels à base de GNF présentent une cinétique de dégradation relativement lente (deux mois), que ce soit *in vitro* ou *in vivo*. Cette lente cinétique est compatible avec les exigences de l'ingénierie tissulaire osseuse dans lesquelles la dégradation de l'hydrogel doit être couplée à son remplacement progressif par du tissu nouvellement formé[122].

Cependant, cet hydrogel ne permet pas l'adhésion des hASCs lorsqu'elles sont dispersées au sein de celui-ci. Or il a été montré que les ostéoblastes doivent adhérer pour se développer et produire de la matrice extracellulaire osseuse. Une précédente étude démontre que des agrégats d'hASCs se développent et se différencient normalement lorsqu'ils sont ensemencés dans le gel à base de GNF.

Lorsque les cellules sont ensemencées en agrégats dans l'hydrogel puis implantés chez la souris, ces agrégats de cellules présents dans le gel survivent pendant plusieurs semaines. De plus, cet hydrogel stimule la différenciation ostéoblastique des hASCs organisés en agrégats, en l'absence de facteur ostéogénique[123].

Ce gel ne libère pas de composés qui seraient toxiques pour les hASCs. De faibles concentrations de GNF solubilisées dans des milieux de culture cellulaire n'entrainent pas d'effets cytotoxiques. Ainsi les hydrogels à base de GNF sont cytocompatibles[121].

Cette étude révèle de nombreuses propriétés intéressantes offertes par ce biomatériau biocompatible, injectable, biodégradable et qui se montre ostéoinducteur en l'absence de facteur de différenciation, dans certaines configurations. Ces propriétés en font un nouveau type d'hydrogel susceptible de convenir pour des applications en ingénierie tissulaire osseuse.

Une nouvelle approche consiste à ajouter à cet hydrogel une matrice permettant l'adhésion et la croissance des cellules. Cette matrice peut être faite d'hydrogel de collagène, reconnue pour ses propriétés favorables à la culture cellulaire en trois dimensions[107] pouvant offrir aux intégrines des cellules des sites d'ancrage afin de se lier au matériau pour fournir un microenvironnement adéquat à la survie et la différenciation cellulaire[124].

2.1.4. La protéine de morphogénèse osseuse BMP-2

La protéine de morphogénèse osseuse BMP-2 a été initialement identifiée comme une protéine qui induit la formation osseuse ectopique lorsqu'elle est implantée dans le tissu musculaire [125]. La BMP2 est un puissant inducteur de la différenciation de divers types

cellulaires en ostéoblastes et de la formation osseuse. En effet, elle est impliquée dans l'engagement des cellules souches multipotentes vers la lignée ostéoblastique[126]. Plusieurs études ont démontré que la BMP2 induit ou favorise l'expression des marqueurs de différenciation des ostéoblastes tels que la phosphatase alcaline (ALP), la présence de collagène de type I et d'ostéocalcine dans diverses cellules[127]. La BMP-2 est utilisée en routine clinique dans le traitement de certaines pathologies osseuses, son utilisation est approuvée par la FDA depuis 2002. Pour nos expérimentations, de la BMP-2 recombinante humaine dérivée de la bactérie Escherichia Coli a été utilisée.

2.2. Les stratégies de conception et développement des produits d'ingénierie osseuse

L'objectif de ces travaux de thèse est de concevoir, développer et étudier de nouveaux produits pour le comblement et la régénération du tissu osseux à partir d'hydrogels. Pour y parvenir deux stratégies seront mises en place. Premièrement en développant une matrice d'échafaudage composite qui est formée de deux hydrogels pour la libération de cellules souches par injection sur site. La deuxième stratégie est de proposer un système de libération prolongée de facteurs de croissance favorisant le recrutement et la différenciation cellules ayant la capacité de promouvoir la réparation osseuse sur des modèles de lésion osseuse de taille critique chez la souris.

Ce travail de thèse s'intègre dans la continuité des travaux réalisés au laboratoire Biotis qui ont permis le développement d'hydrogels physique thermosensibles pour l'ingénierie tissulaire osseuse ainsi que les travaux du LBB portant sur l'extraction du collagène pour la fabrication d'hydrogel aux excellentes propriétés biologiques pour l'ingénierie tissulaire.

Dans ce contexte, les travaux de thèses s'articuleront autour de trois chapitres principaux :

1. La création de microenvironnement aux propriétés physiologiques adéquates pour la régénération du tissu osseux à partir d'hydrogels. Ce chapitre est présenté sous forme d'une revue de la littérature.

2. Le développement et l'étude d'un nouvel hydrogel composite qui combine les propriétés biologiques du collagène et les propriétés mécaniques d'un échafaudage supra moléculaire pour l'injection de cellules souches. Ce chapitre concerne la

conception et la caractérisation d'un hydrogel composite, l'étude des propriétés de ce matériau est réalisée *in vitro* puis *in vivo* en site sous cutané chez le petit animal.

3. Le développement et l'étude d'un système à libération prolongée de facteurs de croissance (BMP-2) à partir d'un hydrogel. Après avoir réalisé la conception d'un système à libération prolongée de BMP-2 à partir d'un hydrogel de GNF celui-ci sera étudié *in vitro* et *in vivo* en site de lésion osseuse chez la souris.

3. Cellularizing hydrogel-based scaffolds to repair bone tissue: How to create a physiologically-relevant microenvironment?

Mathieu Maisani[1,2], Daniele Pezzoli[1], Olivier Chassande[2*], Diego Mantovani[1*]

[1]*Lab. for Biomaterials & Bioengineering CRC-I, Dept. Min-Met-Materials Engineering & Research Center CHU de Québec, Laval University, Québec City, Canada.*

[2]*Laboratoire BIOTIS, INSERM U1026, Université de Bordeaux, Bordeaux, France.*

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3.1. Résumé

L'ingénierie tissulaire est une alternative prometteuse aux autogreffes ou aux allogreffes pour la régénération des lésions osseuses de taille critique. De nombreux biomatériaux présentant différents degrés de sophistication peuvent être utilisés sans l'apport de cellules exogènes dans le cas de plusieurs indications thérapeutiques pour stimuler les processus de régénération osseuse par le tissus hôte. Cependant, lorsque les ostéoprogéniteurs ne sont pas disponibles au sein du tissus hôte, il est nécessaire de fournir des cellules exogènes ayant la capacité de se différencier en cellules osseuses. Ces cellules doivent coloniser la lésion osseuse et participer à la formation du nouveau tissu osseux. Pour y parvenir, les cellules doivent survivre, rester localisées au sein de la lésion, proliférer et se différencier en ostéoblastes matures. Un défi à relever concerne l'alimentation en oxygène et en nutriments des cellules greffées. En effet, l'absence transitoire de vascularisation lors de l'implantation est une menace à la survie des cellules au sein de la zone d'implantation. Ainsi, plusieurs stratégies peuvent être mises en place pour améliorer la survie cellulaire malgré la faible quantité d'oxygène et de nutriments, et développer rapidement un réseau vasculaire au sein de la zone lésée. Ces stratégies impliquent d'utiliser des échafaudages conçus pour créer un microenvironnement adapté pour que les cellules survivent, prolifèrent et se différencient *in vitro* et *in vivo*. Les hydrogels sont une classe de biomatériaux qui peuvent, de par leurs propriétés, être facilement être ensemencées de cellules et être implanté pour combler les lésions osseuses afin de favoriser la régénération des tissus osseux. En jouant sur leur composition et les techniques de préparation, il est possible d'obtenir des systèmes biocompatibles dotés de propriétés chimiques, biologiques et mécaniques offrant des approches prometteuse pour une utilisation en ingénierie tissulaire osseuse. Cependant, seule une bonne combinaison de l'échafaudage à base d'hydrogel et de cellules, accompagnée éventuellement de facteurs de croissances ensemencés au sein de l'hydrogel, peut conduire à des résultats satisfaisants pour la régénération des lésions osseuses. Cette revue de la littérature présente les stratégies utilisées pour concevoir des systèmes d'hydrogels cellularisés appliquées à la régénération osseuse, en identifiant les paramètres clés des microenvironnements créés par l'utilisation d'hydrogels.

Mots-clés

Cellules souches, Hydrogels, Ingénierie tissulaire osseuse, Microenvironnement

Abstract

Tissue engineering is a promising alternative to autografts or allografts for the regeneration of large bone defects. Cell-free biomaterials with different degrees of sophistication can be used for several therapeutic indications, to stimulate bone repair by the host tissue. However, when osteoprogenitors are not available in the damaged tissue, exogenous cells, with an osteoblast differentiation potential must be provided. These cells should have the capacity to colonize the defect and to participate in the building of new bone tissue. To achieve this goal, cells must survive, remain in the defect site, eventually proliferate, and differentiate into mature osteoblasts. A critical issue for these engrafted cells is to be fed by oxygen and nutrients: the transient absence of a vascular network upon implantation is a major challenge for cells to survive in the site of implantation, and different strategies can be followed to promote cell survival under poor oxygen and nutrient supply, and to promote rapid vascularization of the defect area. These strategies involve the use of scaffolds designed to create the appropriate micro-environment for cells to survive, proliferate and differentiate *in vitro* and *in vivo*. Hydrogels are an eclectic class of materials that can be easily cellularised and provide effective, minimally invasive approaches to fill bone defects and favor bone tissue regeneration. Furthermore, by playing on their composition and processing it is possible to obtain biocompatible systems with adequate chemical, biological and mechanical properties. However only a good combination of scaffold and cells, possibly with the aid of incorporated growth factors, can lead to successful results in bone regeneration. This review presents the strategies used to design cellularised hydrogel-based systems for bone regeneration, identifying the key parameters of the many different micro-environments created within hydrogels.

Keywords

Stem cells, Hydrogels, Bone Tissue Engineering, Micro-environment

3.2.Introduction

Severe bone lesions cause hundreds of millions of surgical procedures each year around the world. Bone is a dynamic and vascularized tissue that has the ability of naturally healing upon damage. Nevertheless, in the case of large defects (such as in non-union fractures[129], maxillofacial trauma[130,131], tumor ablations[132,133], intervertebral disc injury or degeneration[134,135]) this potential is impaired and surgical procedures including the use of autografts, allografts or grafting of exogenous biomaterials are necessary. These grafted materials must ensure mechanical stability and provide the appropriate environment for efficient healing[136,137]. These approaches present several limitations: (i) autografts may involve tissue morbidity, moreover the availability of donor tissue is limited; (ii) allografts cause an important risk of infection and immunogenic rejection mechanisms; (iii) solid biomaterials such as metal or ceramic implants do not easily fit the size and shape of the defect[138]. Although recent advances in 3D printing of solid materials have enabled the fabrication of size and shape-controlled materials, their surgical implantation to fit the morphology of the damaged site is far from easy. In this context, new classes of biomaterials for bone healing are the focus of much research. A promising strategy for the regeneration of bone is bone tissue engineering (BTE), based on the use of 3D matrices (scaffolds) to guide cellular growth and differentiation and to promote the deposition of new bone tissue[139].Hydrogels are among the most promising biomaterials in BTE applications since they are very flexible materials that allow several different properties to be targeted for specific applications and they can be formulated in order to be implantable with minimal invasive procedures. In fact, ideally hydrogels should be injectable. In contrast to rigid scaffolds, hydrogels can establish tight contacts with the host tissue, limiting fibrosis and favouring osteoconductivity. The only limitation of hydrogels is their low stiffness, which does not allow their use for the repair of load-bearing lesions, such as large fractures of long bones. Instead, hydrogels rather appear as lesion filling materials. Hydrogels are hydrophilic polymeric 3D networks which can contain and/or release in a controlled fashion cells for tissue regeneration and/or bioactive molecules such as growth factors[136].The cells encapsulated in hydrogel systems can exert two types of effects. They can directly take part as building blocks in tissue regeneration, and in such case their long-term survival is required. Alternatively, they can stimulate host responses, ultimately favoring tissue repair[140]. In this latter case, transient persistence of these cells may be sufficient. Whatever the mechanisms, the choice of the appropriate progenitor cells and of appropriate culture conditions prior to incorporation in the hydrogel scaffold are key issues for the efficiency of BTE products.

The present review, after describing the physiology of bone tissue and its healing mechanisms, is intended to provide a critical overview of the cells employed for bone tissue regeneration and of hydrogel-based scaffolds as optimal, potentially injectable, physiologically-relevant micro-environments for the survival, recruitment, proliferation and differentiation of bone cells in BTE applications. Relevant examples in the literature will be examined attempting to determine the key parameters which may influence cell behaviour and fate, at each of the many different steps of the preparation of tissue engineering hydrogel-based constructs.

3.3. Bone physiology and healing mechanisms

3.3.1. Bone structure

Bone is a connective tissue that can be considered as a composite cellularised living material typically composed of an inner spongy bone, often named cancellous bone or trabecular bone, and an outer compact bone also defined as cortical bone, whose relative mass ratio is 20%-80% in the whole skeletal system[2].

Cortical bone is composed of osteons, or haversian systems, cylindrical structures around 200 μm in diameter, with an inner channel (the haversian canal) containing blood vessels surrounded by concentric lamellae of mineralized matrix among which small cavities called lacunae are present, each containing an osteocyte. A network of small channels (canaliculi) connects the lacunae and the harversian canals, allowing cell-cell interactions and favouring exchange of nutrients and metabolites.

The honeycomb-like network of trabeculae forming cancellous bone also has a lamellar organization but internal canals and blood vessels are missing. The trabecular network in fact is filled with marrow, a tissue composed of blood vessels, nerves and several cell types, from which trabecular osteocytes receive nutrients.

Bone extracellular matrix (ECM) is characterized by two phases: an inorganic mineral component and an organic protein phase. The inorganic component provides stiffness to the bone and is mainly composed of hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] crystals, even if calcium carbonate, calcium fluoride and magnesium fluoride are also present, and serves as reservoir for the homeostasis of ions, containing 99% of calcium and 88% of phosphate of the human body[141].

The organic component is mainly composed of a network of type I collagen triple helices organised in fibrils (ca 90%), the remaining part being non-collagenous proteins such as glycoproteins, proteoglycans and growth factors. The structural role of the organic ECM

is twofold: regulating the nucleation and direction of HA crystals and thus the shape of the bone and providing ductility and fracture toughness. In addition, the inorganic ECM is a reservoir for growth factors and cytokines involved in bone remodeling and reparation.

Among non-collagenous proteins, approximately 10% is constituted of proteoglycans (PG), macromolecules composed of strongly hydrophilic negatively charged long carbohydrate chains (glycosaminoglycans, GAGs, mainly chondroitin sulphate, dermatan sulphate, chondroitin sulphate, keratan sulphate and hyaluronic acid) covalently linked to a core protein[10]. PGs form a highly hydrated swelled gel-like matrix whose main role is to provide resistance to compressive stress. In addition PGs function as growth factors binding and storage agents and as regulators of collagen fibrillogenesis[11]. Bone ECM glycoproteins include alkaline phosphatase (ALP), osteopontin, bone sialoprotein and osteocalcin, all involved in the mineralization process.

3.1.1. Bone remodeling

Bone growth, modeling and remodeling are lifelong processes meant to guarantee tissue size and shape adaptation, structural integrity and regulation of mineral homeostasis. Bone growth occurs mainly during childhood and adolescence; modeling consists in the gradual transformation of bone shape in response to the applied mechanical forces; finally, bone remodeling is the continuous process by which bone tissue is renewed to maintain its integrity and strength and to control mineral homeostasis.

Bone remodeling is tightly regulated by the orchestral action of an ensemble of multiple cell types arranged within temporary bone-remodeling compartments known as basic multicellular units or bone metabolic units (BMU)[29]. Remodeling is initiated by remodeling signals (activation phase) that can be hormones such as parathyroid hormone (PTH), secreted to maintain calcium homeostasis, or mechanical stimuli detected by osteocytes, inactive osteoblasts with low metabolic function located in bone lacunae that serve as stress and strain sensors and express paracrine signals for active osteoblasts and osteoclasts (e.g., the inhibitory osteoclastogenesis signal transforming growth factor-β, TGF-β) thus directing bone turnover. However, it is osteoblasts that, in response to remodeling signals can produce osteoclastogenesis cytokines and directly recruit osteoclast precursors and promote their proliferation and differentiation into multinucleated osteoclasts that begin the bone resorption phase. Osteoclasts derive from mononuclear precursors of the hematopoietic lineage that, upon stimulation by cytokines produced by osteoblasts, undergo fusion forming large multinucleated cells[19]. Bone resorption is achieved by secretion of H^+ through membrane proton

pumps that create an acidic environment, with a pH as low as 4.5, in the resorptive pit (Howship lacuna) that dissolves the mineral component of the matrix while kathepsin K and other enzymes are released to break down the organic ECM. Then osteoclasts undergo apoptosis and are substituted by mononuclear cells of still unclear phenotype (reversal cells) that conclude the resorption phase and prepare the surface for the deposition of new matrix[28]. The resorptive pit is then occupied by the osteocytes released from the resorbed matrix, mesenchymal stromal cells (MSCs) and preosteoblasts that are recruited from the medullary cavity or the periosteum, a fibrous membrane covering the external surface of bones populated by progenitor cells, by mature osteoclasts, reversal cells and signals liberated from the degraded bone matrix. Mature osteoblasts are generated by differentiationof progenitor cells by growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF) and TGF-βand are then responsible for the synthesis of new bone ECM.As osteoblasts end depositing new matrix, three main possible fates are possible for them: (i) remaining embedded in the newly formed mineralised tissue and transforming into osteocytes; (ii) undergoing apoptosis; or (iii) becoming bone lining cells, quiescent osteoblasts with a flat morphology that populate non-remodeling bone surfaces[17].

A comprehensive description of the cellular and molecular mechanisms coordinating the different phases of bone remodeling is beyond the purposes of this review and it has been extensively reviewed elsewhere[28,29].

3.1.2. Bone healing

Bone has an intrinsic ability to repair itself. Bone healing processes are not fully understood but their understanding is the key to the design and development of new effective strategies for the treatment of non-healing bone defects. When fractures occur, locally, the skeletal integrity is lost and the bone vascular network is disrupted leading to impaired nutrient and oxygen flow and affecting the marrow structure[32]. Then the tissue regeneration process begins following three main phases: the inflammation (reactive) phase, the reparative phase and the remodeling phase[32].

In the early inflammation phase a blood clot (haematoma) is locally formed and growth factors (e.g., insulin-like growth factor I (IGF-I) and platelet derived growth factor (PDGF)) and cytokines are released to attract and regulate the action of monocyte–macrophages and osteo-chondroblast precursor cells. Then the recruited immune cells secrete signaling molecules (e.g., FGF, tumor necrosis factor- α (TNF-α), vascular endothelial growth factor (VEGF), TGF-β, interleukin-1 and -6 (IL-1 , IL-6)) that stimulate ECM synthesis and angiogenesis and chemotactically attract other inflammatory cells and

mesenchymal cell precursors (mainly originating from the periosteum) that proliferate and differentiate into chondrogenic and osteogenic lineages[34] finally forming a transient granulation tissue.

In the reparative phase the so called fracture callus is generated by one of the two following ossification processes: endochondral ossification and intramembranous ossification. In endochondral ossification chondroblasts deposit a cartilaginous callus bridging and stabilizing the fracture site that is then calcified, vascularised and gradually substituted by osteoblasts with woven bone, mechanically weak and characterized by a random organization of the collagen fibers. In intramembranous ossification both compact and trabecular bone are directly synthesised by osteoblasts without the intermediary cartilage deposition phase. This process is mainly limited to the subperiostal regions adjacent to both the ends of the fracture[35] and the bone marrow, characterised by a functional capillary network and high O_2 tension[34].

Finally, in the remodeling phase, the fracture callus is converted into new bone tissue with a lamellar structure and an inner medullary cavity, thus finally fully restoring the biomechanical properties of the bone[35]. Similarly to bone remodelling in intact bones, the remodelling phase of the bone healing process is based on BMUs and consists in a combination of callus resorption by osteoclasts and bone deposition by osteoblasts and may take years to achieve the fully repaired bone.

For a complete description of the biology of fracture healing, involving the tight coordination of several cell types and changes in the expression of thousand genes, the reader is referred to specific comprehensive reviews[34,35].

3.1.2.1. MSCs in bone healing

MSCs play a pivotal role in bone healing by differentiating into chondroblasts and osteoblasts that deposit the fracture callus in the reparative phase[142]. They are mainly recruited from the bone marrow and the periosteum, even if a systemic recruitment of MSCs circulating in the blood is also possible[143]. The process regulating SMC recruitment in the site of injury is still not completely understood, since it is often difficult to clearly discriminate effects on recruitment, proliferation and differentiation. However, it is generally agreed that they migrate along chemical gradients of potent chemokines and growth factors by chemotaxis and stromal cell-derived factor-1 (SDF-1) is currently the most recognised recruitment signal[144].

Also, the molecular mechanisms governing proliferation and differentiation of MSCs are still not fully elucidated. Several signaling pathways are involved in parallel,

encompassing FGF, BMPs, Wnt and Notch signaling, but also physiologic stimuli such as mechanical strain and hypoxia[142]. The combination of these differentiation signals, during bone healing, can lead to the production of osteoblasts or chondrocytes that finally accomplish bone formation. In addition, MSCs, can play an indirect trophic role in fracture healing by secreting cytokines and growth factors thus contributing in the recruitment of other cells, in the stimulation of vascularization and in the modulation of immunological responses[142,145].

3.2. Hydrogels: suitable micro-environments for BTE

Some large fractures or lesions caused by loss of large amounts of trabecular bone cannot self-heal and require biomaterials either as substitute or as filler to restore the mechanical properties of the damaged organ[146]. In these situations, regeneration of damaged bone necessitates either an osteoconductive biomaterial, which will enable good osteointegration, or an osteoinductive system, which will enable the recruitment and differentiation of host cells. In some instances, osteoblast progenitors are not available in the vicinity of the lesion, and exogenous stem cells may be implanted. This therapeutic approach is known as tissue engineering, in which a scaffold is associated with stem cells and growth factors to be implanted in severe lesions, to promote efficient formation of new vascularized bone, with biological and mechanical characteristics as close as possible to those of native bone.

Biomaterials for bone repair must be able to provide temporary structural and mechanical support to the tissue regenerating cells which will colonize them, allowing their proliferation, possibly the differentiation in suitable cell types and finally the synthesis of a mineralized bone matrix that will replace the scaffold itself. As described in the previous section, the bone micro-environment is complex, consequently several properties are required to the 3D scaffold material in order to favor an adequate regeneration of the bone tissue[147].

First, a scaffold material must be biocompatible, generally meaning that upon implantation it must not cause an important deleterious inflammatory reaction or other adverse topic or systemic effects and should not be toxic for the recipient tissues and the cells it can harbor[148],[149]. The material should be bioactive, particularly at the interface with the host tissue allowing (i) the establishment of bonds and connections with the surrounding bone and thus a rapid osseointegration and (ii) the colonization of the scaffold by osteoprogenitor and differentiated bone cells that can promote the deposition of new bone tissue. In this context, scaffolds for bone regeneration should satisfy three main properties: osteoconductivity, osteoinductivity and osteogenicity.

- Osteoconductivity is the ability of the material to favor bone growth at the biomaterial/host interface that is on the external and internal surfaces of the scaffold.

- Osteoconduction is perpetrated by stimulating the adhesion and migration of cells from the surrounding bone within the material and the deposition of new bone tissue [150]. It is dependent on the physical, chemical and structural (e.g., porosity) properties of the scaffold but also mechanical properties, biocompatibility, biodegradability and hydrophilicity will influence it[136].

- Osteoinductivity, is the bioactive ability of the scaffold to recruit stem cells and promote their differentiation towards osteogenic lineages, thus inducing bone regeneration.

- Osteoinduction can be stimulated by material chemical properties, by its structure (macrostructure, microstructure and nanostructure) or by the presence of osteoinductive growth factors such as BMPs [151],[152].

- Osteogenicity implies the presence of osteoprogenitor cells inside the graft (e.g., autografts) or the scaffold material and their proliferation in order to create a cellular environment prone to osteogenesis [153].

Hydrogels are three-dimensional strongly hydrophilic polymer networks which can absorb huge quantities of water and that mimic the characteristics of the ECM of native tissues, providing cells with a temporary mechanical support while guaranteeing adequate nutrient and gas exchange[154]. This provides an ideal micro-environment for cellular proliferation and differentiation, thus allowing bone cells encapsulated/migrating in the hydrogel to grow and secrete new ECM for restoration of damaged bone tissue[155].Thanks to all these advantages hydrogels are increasingly considered as the option of choice for bone regeneration. In addition, hydrogels can possibly be loaded with bioactive molecules, osteoconductive/osteogenic growth factors, or with cells and injected in the site of morbidity before gelation[156]. These injectable hydrogels permit less invasive surgical procedures, with respect to hard scaffolds, regardless of the shape of the bone lesion since they can easily fill also irregularly shaped defects[157].

Owing to the plethora of advantages offered by hydrogels, this section will focus on the methods of preparation, the properties and the composition of hydrogels as systems providing a physiologically relevant environment for cell adhesion, proliferation and differentiation for BTE strategies.

3.2.1. Methods of preparation of hydrogels

The mechanical (visco)elastic behaviour and the extent of swelling of hydrogels depend on the balance between the osmotic forces that promote water inflow and the cohesive forces that resist the deformation of the polymeric 3D network and impart it mechanical reinforcement and stress-resistance. Therefore, the swelling ratio strongly depends on the chemical properties of the polymeric components (i.e., hydrophilicity) and on the type and extent of crosslinking[158]. It is thus clear that by playing with the method of preparation and with the parameters of the crosslinking reaction it is possible to tune the final properties of hydrogels.

For the preparation of hydrogels hydrophilic polymers are crosslinked either through covalent bonds or via physical intra- and intermolecular interactions. The main methods of preparation of hydrogels, together with their main advantages and limitations are summarized in Table 1.

3.2.1.1. Physical crosslinking

In physically crosslinked hydrogels the junctions among polymeric chains are mediated by transient non-covalent interactions such as ionic interactions, hydrogen bonds and hydrophobic effects or simply by chain entanglement[159]. These processes allow to avoid the addition of cytotoxic initiators and chemical crosslinkers and to employ mild conditions of preparation (e.g., pH and temperature) thus improving the cytocompatibility of the hydrogels and possibly permitting the incorporation of cells prior to gelation (e.g., type I collagen gels[115]). Physical crosslinking techniques employed for the preparation of hydrogels rely on (i) ionic crosslinking, where polyelectrolytes form hydrogels in the presence of multivalent ions of the opposite charge that create bridges between pairs of charged functionalities present along the backbone of the polymeric chains[160], such as in the gelation process of alginate and pectin by calcium ions[161]; (ii) hydrogen bonding, such as in gelatin based hydrogels[162]; and (iii) hydrophobic association, occurring when the hydrophobic portions of amphiphilic polymers in aqueous milieu aggregate as the temperature is increased above their transition temperature[163], as recently described for the injectable thermosensitive copolymer poly(ethylene glycol)–poly(serinol hexamethylene urethane) (ESHU) that forms hydrogels at body temperature and has been successfully used for bone marrow MSC (BMSC) transplantation[164].

The main drawbacks of physical hydrogels are generally the low mechanical properties, deriving from the weakness of the secondary forces involved in crosslinking, that limit their application to non-load-bearing sites. In addition, the stability in physiological

environments could be an issue given that premature disassembly of the hydrogels can prevent effective cell engraftment.

3.2.1.2. Covalent crosslinking

Covalently crosslinked hydrogels overcome the limitations of physical hydrogels related to stability, dwell time after implantation and, partially, mechanical properties. Many different chemistries have been employed for covalently crosslinked hydrogels among which free radical polymerization, click chemistry, Michael-type addition, photocrosslinking, enzymatic crosslinking[134,160]. Generally, these systems are composed of polymeric chains bearing reaction sites for 3D network expansion under specific physical and chemical conditions. However, these approaches are suitable for tissue engineering/regeneration only if the employed possibly toxic reagents (precursors, initiators, crosslinkers) can be completely removed before cell addition or implantation. In addition, most of covalently crosslinked hydrogels do not allow direct incorporation of cells inside the hydrogel, making necessary to seed the cells on the surface and, provided that there is a suitable open macroporosity, let them migrate inside the scaffold. Moreover, chemical functionalization and crosslinking of the starting polymer chains can thoroughly affect their chemistry and then their biological properties, especially for naturally derived materials.

An interesting approach to covalently crosslink polymers in hydrogel systems is to use enzymes. In this approach, the crosslinking reaction proceeds under physiological conditions making the systems highly cytocompatible, injectable for *in situ* gelation and suitable for direct cell encapsulation. In addition, the properties of the resulting hydrogels can be modulated by controlling the concentration and the activity of the enzymes. One of the most employed enzymes is transglutaminase. Transglutaminases are enzymes that catalyse the formation of isopeptide (amide) bonds between proteins in processes such as blood clot formation. These class of enzymes has been used to crosslink hydrogels based on different proteins, mainly collagen[165] and gelatin[166], that were demonstrated to be non-cytotoxic and suitable for cell encapsulation[167,168].

For the in-depth description of crosslinking techniques in hydrogels the reader is referred to specific comprehensive reviews[134,159].

3.2.2. Controlling hydrogel formation by 3D printing technologies

For many years, hydrogels have been formed as bulks or particles, without any control of the organization of the scaffold. Likewise, inclusion of cells or bioactive factors was achieved by simple blending procedures or surface seeding. The recent development of

several 3D printing technologies has opened the way to new possibilities of better controlling the pattern of gels, in particular structure and porosity, from the macroscopic to the microscopic scale, enabling the design of complex, heterogeneous products comprising materials, cells and growth factors with a controlled organization[169]. Three types of printing technologies are currently used: inkjet, extrusion, and laser-mediated printing, allowing different resolutions[169]. Different compounds have been used to produce hydrogels by extrusion and inkjet techniques, such as collagen[170], alginate[171], silk fibroin[172], , or synthetic polymers such as polyethylenglycol, acrylates, polyions complex hydrogels [173], or polycaprolactones (PCL)[174]. These technologies also allow the production of interpenetrating networks consisting in the mixture of different polymers resulting in improved overall mechanical properties[171]. The possibility to perform *in situ* gel formation upon printing, by physical agents such as ultraviolet light (UV) or temperature, or chemical agents such as pH or radicals[175], has been shown to greatly improve the accuracy and stability of the printed pattern[176]. In addition to controlling gel structure, it is also possible to control cell patterning, using specific natural matrices as bioink for cell printing and separate nozzles to print the gel-forming solution and the cell-containing matrix separately. The combined control of material and cell patterning offers multiple applications for the repair of many tissues, including bone[177,178]. However, whereas many protocols have shown excellent *in vitro* properties such as cytocompatibility, well-controlled cell distribution, viability over extended periods of time in culture and sometimes improved osteoblast differentiation, the real benefit of 3D printing technologies for bone regeneration remains to be demonstrated by further *in vivo* studies.

3.2.3. Tailoring hydrogel properties for cell incorporation and bone tissue regeneration

Several material properties must be tuned in parallel to obtain physiologically-relevant microenvironments for bone cell incorporation, survival and differentiation, for bone ECM deposition and for the recruitment of cells involved in the complex bone regeneration process.

The mechanical properties of hydrogels for BTE are important since scaffolds are supposed to bear loads while promoting the tissue regeneration. In general, hydrogels feature poor mechanical properties, compared to the bone tissue this limiting their application to non-load or low-load bearing sites[155]. However, secondary materials such as HA nanoparticles, bioglasses, carbon nanotubes and nanofibers can be

incorporated into hydrogels, obtaining composite materials with appropriate mechanical performances, as recently reviewed by Tozzi *et al.*[136] and Butcher *et al.*[179].

In addition, material stiffness can influence cell behaviour in terms of adhesion, proliferation, migration and differentiation[157], with higher matrix rigidity associated with increased osteogenic differentiation of osteoblast progenitor cells and tissue mineralization[180]. Hydrogel stiffness can be tailored by playing on several preparation processing parameters such as polymer molecular weight, concentration, and type and degree of crosslinking, however this will affect also other relevant properties of the system, in particular porosity, permeability and cytocompatibility[136], making necessary to finely tune the material design to obtain adequate combinations of mechanical, structural and biological properties.

When considering hydrogel mechanical properties, polymer degradation should be also taken into account. In fact, ideally, the scaffold should degrade at a rate compatible with new bone formation, so that the mechanical stability of the site of injury is maintained[181], and the degradation products should be non-toxic for the cells present in the regenerating and surrounding tissues. Degradation usually occurs by (enzymatic) hydrolysis of ester linkages and degradation rate can be controlled through the chemistry and length of the polymer backbone and of the crosslinkers, through the crosslinking density and by the introduction of degradation sites susceptible to cleavage by enzymes such as metalloproteinases[182].

Porosity of hydrogels strongly influences the fate of osteogenic progenitor cells and thus their ability to mediate new bone formation. In general, hydrogels for BTE should have a high and open interconnected porosity to maximise surface/volume ratio and thus cell-biomaterial interactions, facilitate cell seeding and colonization, allow the appropriate supply of oxygen and nutrients from the surrounding tissues and permit neovascularization. Porosities higher than 90% are often chosen for BTE scaffolds[14] and pore size bigger than 200 μm is generally considered appropriate to stimulate osteogenesis, osteoinduction, osteoconduction and osteogenic progenitor cell differentiation[158,183–186].

Porosity can be tailored by playing with the degree of crosslinking (higher crosslinking corresponds to reduced porosity) and porogen materials can be introduced during hydrogel preparation to finely control the final structural properties of the scaffold[187]. Recently Wang et al. proposed uncrosslinked gelatin microspheres as porogen agent. Gelatin microsphere can be incorporated in the hydrogel at room temperature but they

dissolve in non-cytotoxic products at 37°C, allowing to control porosity and pore size in cell-laden hydrogels without affecting cell viability[187].

However, it must be noticed that scaffold mechanical strength decreases with porosity and pore size, therefore these parameters should always be balanced to guarantee the preservation of the mechanical stability of the hydrogel.

The possibility to directly and uniformly encapsulate cells during preparation is a desired property of hydrogels for BTE, since it bypasses the time-consuming cell-seeding/colonization steps necessary for the cellularization of prefabricated scaffolds often associated with limited and unequal cell infiltration. In addition, these systems are often also injectable, allowing minimally invasive administration routes and to easily fit the defect, thus providing a superior configuration for osteoconduction and vascularization from the surrounding tissues[134]. For direct cell encapsulation, the gelation process must occur in cell compatible conditions (pH, temperature, osmolarity). When covalent crosslinking is employed, often the chemical reactions have cross-reactivity with cell components and it is therefore necessary to use nontoxic crosslinkers (e.g., genipin[188]) and initiators (e.g., lithium acylphosphinate salt for photopolymerization) and to investigate the compatibility of the crosslinking strategies with cells, as recently reviewed by Caliari and Burdick[189]. These issues limit the number of suitable crosslinking strategies and compel to develop specific optimized procedures to preserve the viability of encapsulated cells. Thermosensitive hydrogels are an interesting class of materials that undergo gelation above a transition temperature called lower critical solution temperature (LCST), due to hydrophobic association. Thermosensitive hydrogel-forming polymers are amphiphilic copolymers whose LCST can be tuned by changing the molecular weight of the hydrophobic and hydrophilic portions. When the LCST is at values around physiological temperature, these systems are suitable for cell encapsulation and can be injected into the body for *in situ* formation[190]. Poly(N-isopropylacrylamide) (PNIPAAm) and Pluronic® are typical examples of thermosensitive polymers that have been used for bone cells encapsulations[191,192].

Biomimetic approaches aim to introduce bioactive molecules in the hydrogel structure to promote osteoconductivity, osteoinductivity and osteogenicity. First of all, cells used in BTE, in particular MSCs, are strongly adhesion dependent, they need to adhere to the substrate to survive, proliferate and differentiate and, when not properly attached, they may undergo anoikis, a form of apoptosis occurring in anchorage-dependent cells when they detach from ECM[193]. Natural polymers such as type I collagen inherently possess

bioactive motifs that can guide cell adhesion, proliferation and differentiation and tissue regeneration[194]. When inherent bioactivity is missing, biological cues can be incorporated by covalent grafting or inclusion during fabrication. However, biological moieties grafting should be limited to avoid affecting the structural and mechanical properties of the resulting hydrogel. The natural cell binding ligand arginine-glycine-aspartate (RGD), found in collagen, fibronectin and other ECM proteins, is the most widely employed signal to improve cell adhesion. Interestingly, it has been recently reported that by controlling the distribution of RGD on hydrogels by nanopatterning it is possible to maximize its beneficial effects on adhesion, survival and differentiation of MSCs[195,196]. This strategy is promising for prefabricated hydrogels and can be exploited for the investigation of the influence of RGD density and spatial distribution on *in vitro* MSC differentiation but it is hard to translate to hydrogels for BTE applications, especially to hydrogels for direct cell encapsulation, where only the grafting degree can be controlled during synthesis.

As recently reviewed by Nyberg and colleagues[197], growth factors such as BMP-2, TGF-β, FGF and IGF can be incorporated to control MSC differentiation and the recruitment of progenitor cells from the surrounding systems, aiming to mimic the signalling events occurring during bone healing, but also alternative small osteogenic molecules such as melatonin, resveratrol and purmorphamine have recently demonstrated promising activity in BTE strategies[198]. However, fine tuning the properties of hydrogel-based scaffolds for the combined transplantation of cells and controlled delivery of osteogenic molecules is challenging due to the different characteristics required for the two approaches (additional drug delivery particles could be necessary) and it is not clear yet if the synergistic effects are significant[199,200].

It is worth to note that, unfortunately, to date the majority of the studies on bone progenitor cell differentiation in hydrogels have been performed in *in vitro* settings, whose predictivity toward *in vivo* outcomes is still debated[156]. In addition, controlling separately structural, mechanical, chemical and biological properties of hydrogels is challenging since, as described above, usually a change in processing parameters strongly influences these properties all at once, for example, increasing crosslinking density to improve the strength of the scaffold yields reduced pore size and longer degradation times and can affect the cytocompatibility necessary for cell encapsulation. Consequently, also the investigation of the effects of single parameters on cell behaviour is not trivial, and the hydrogel system properties as a whole must be taken into account in the design of hydrogels for BTE.

Tableau 2 : Méthodes de preparation des hydrogels

Type of crosslinking	Example of material	Advantages	Limitations	Ref.
Ionic crosslinking (physical)	Alginate Pectin	Cytocompatibility Cell encapsulation-injectability	Poor mechanical properties Low stability in physiological environments	[134,155,161]
Hydrogen bonding (physical)	Gelatin	Cytocompatibility Cell encapsulation-injectability	Poor mechanical properties Low stability in physiological environments	[162,163,201]
Hydrophobic association (physical)	ESHU PNIPAAm Pluronic®	Thermoresponsiveness Cytocompatibility LCST tunable at ~ 37°C Cell encapsulation-injectability	Poor mechanical properties	[155,158,163,164]
Free radical polymerization (covalent)	Vinyl monomer-containing/functionalized polymers	Possible thermal-, redox- and photo-initiation Cell encapsulation-injectability Tunable properties	Risk of cytotoxicity	[155,202,203]
Small crosslinkers (covalent)	Polymers with suitable functionalities (e.g., NH_2, COOH, CHO) to react with crosslinking agents	Easiness and versatility Tunable properties	Possible cytotoxicity of the crosslinking agent	[158,163,204]
Direct chain-chain crosslinking (covalent)	Polymers functionalized with reactive functional groups	No toxic crosslinking agents Tunable properties	Risk of cytotoxicity Laborious polymer modification step	[134,163]
Enzymatic crosslinking (covalent)	Protein-based hydrogels (collagen, gelatin, fibrin, using transglutaminase)	Crosslinking occurring under physiological conditions High cytocompatibility Cell encapsulation-injectability Easily tunable properties	Poor mechanical properties	[134,165,168,205]

Table 2 abbreviations. ESHU: poly(ethylene glycol)-poly-(serinol hexamethylene urethane); LCST: Lower Critical Solution Temperature

3.3. Hydrogel forming materials

On the basis of the origin of their components, materials for the preparation of hydrogel scaffolds can be classified in natural and synthetic. However, in order to combine the advantages of these two systems, to overcome their limitation and to obtain optimized properties for tissue engineering, several combinatorial approaches have been also thoroughly implemented[155]. The advantages and limitations of the main materials employed in hydrogel-based BTE are summarized in Table 2, together with relevant examples of their *in vitro* and/or *in vivo* performances.

3.3.1. Natural hydrogels

The main naturally occurring polymers synthesized by living organisms are polysaccharides (e.g., pullulan, alginate, chitosan, etc.) or proteins (collagen, silk, fibrin, heparin, etc.)[157]. In general, natural polymers are biocompatible and they allow cell attachment and proliferation due to their physical and biological properties without causing cytotoxic reactions compared to synthetic hydrogels[157]. The main limitations of these polymer-based matrices are their lack of mechanical strength to support the forces occurring in the bone environment, and, for some of these polymers, a lack of cytocompatibility and osteoconductivity, fast degradation rates, high batch to batch variability and some immunological concerns.

3.3.1.1. Collagen

Type I collagen hydrogels are prepared from collagen extracted from mammals by simple neutralization of the acidic collagen solution[115]. Their thermosensitive nature allows the cells to be incorporated when they are liquid, at low temperature and then to be injected and form a gel *in situ*, at 37°C. Collagen is an important constituent of the bone ECM therefore it offers a number of favorable binding sites for bone cells and it is reported to promote mineralized matrix deposition[183],[206],[207]. These physical and biological properties make collagen a very good candidate for BTE as evidenced by the high number of papers present in literature, although the mechanical properties of collagen-based hydrogels are fairly limited[194],[155],[107]. In addition, recently, collagen gels have been described as optimal scaffolds for the coculture of MSC and endothelial cells, promoting, *in vitro,* the increased expression of both osteogenic and angiogenic markers, with respect to other systems such as alginate gels[208].

3.3.1.2. Alginate

Among natural polysaccharides, alginate is a linear anionic copolymer composed of (1-4)-linked β-D-mannuronate and α-L-guluronate residues that is extracted from marine algae[209]. Usually, alginate hydrogels are formed by cross-linking of the hydrophilic

polymer by an ionic cross-linking agent such as divalent cation (Ca^{2+}) [161]. Alginate is generally employed to encapsulate cells and/or molecules such as small chemicals, proteins and drugs[210]. However, to promote cell adhesion and proliferation, an improvement of the hydrogel composition is necessary such as the chemical grafting of RGD containing peptides on the polymer backbone [161]. As an example, Grellier *et al.* showed that BMSCs can synthesize both *in vitro* and *in vivo* a mineralized ECM in RGD-grafted alginate microspheres[211].

3.3.1.3. Chitosan

Chitosan is a linear polysaccharide consisting of randomly distributed N-acetyl-D-glucosamine and D-glucosamine units linked by β (1→4) glycosidic bonds. Chitosan is obtained by the deacetylation of chitin, one of the main components of the exoskeletons of crustaceans and of the cell walls of fungi. Due to the presence of deacetylated units, chitosan is protonated at slightly acidic pH, and these characteristic confer it many of its peculiar properties such as the ability to form hydrogels by ionotropic gelation and antibacterial activity[209]. In addition, depending on the chemical properties of the used chitosan and on its eventual derivatization, chitosan-based hydrogels can be prepared by chemical cross-linking, allowing to obtain a wide range of mechanical, thermal and biological properties[212] and *in situ* gelation can be achieved by photocrosslinking with UV light or temperature-induced crosslinking[213]. To promote cell adhesion and proliferation, the degree of deacetylation of chitosan should be high and this is also reported to improve the mechanical properties of the obtained hydrogels[214]. In a recent approach, Ding and colleagues deacetylated preformed chitin hydrogels yielding physical crosslinked hydrogels with superior mechanical properties, increasing with the degree of acetilation[215]. The reported mechanical and structural properties are promising but the ability of these systems to promote bone tissue regeneration still need to be investigated.

3.3.1.4. Pullulan

Pullulan is another neutral and non-immunogenic polysaccharide produced from the fermentation of starch by the fungus *A. pullulans*. It is composed by maltotriose units (blocks of three glucose residues connected by α-1,4 glycosidic bonds) connected to each other by α-1,6- glycosidic bonds. To improve the mechanical stability of pullulan, it can be cross-linked by trisodium trimetaphosphate which is reported to be nontoxic[216]. Unfortunately, notwithstanding the hydrophilic nature of pullulan-based hydrogels, they do not support adhesion and spreading of cells. To overcome this problem and enhance biostability pullulan has been combined with other materials such as gelatin or

hydroxyapatite nanocrystals[217] or coated with bioactive proteins such as silk fibroin[218].

3.3.2. Synthetic hydrogels

Synthetic hydrogels offer many advantages over natural polymers including unlimited supply, relative lack of immunological concerns, and much higher reproducibility in terms of physical and chemical properties, which is important for the reproductive manufacture of tissue engineering/regeneration products. In addition, they offer the potential for improved control and tuning of the properties, repeatability and safety[157].

Currently, synthetic polymers have emerged as an important alternative for the production of hydrogel-based scaffolds for BTE[219]. Because of their synthetic nature, the chemical properties of these polymers can be easily tailored *ad hoc* to adapt and modulate their physicochemical properties in order to obtain hydrogels that better mimic the morphology and mechanical properties of native extracellular matrices or to modify their kinetics of biodegradation[220]. In addition, they can be functionalized with bioactive compounds to improve their biomimetic and osteogenic behavior[221]. Among the many synthetic polymers available to create hydrogels only few of them have the properties necessary to be selected as physiologically relevant micro-environments for BTE, such as polyesters (e.g., polyglycolic acid, polylactic acid, polycaprolactone, etc.), polyacrylates, polyethylenglycol (PEG), polyphosphoesters and synthetic peptides.

3.3.2.1. Polyethylene glycol-based hydrogels

Polyethylene glycol (PEG), also known as polyethylene oxide (PEO) is a linear polyether manufactured from ethylene glycol monomers. The name PEG is usually used to indicate polymers with a molecular weight lower than 20 kDa while PEO refers to chains with higher molecular weight. For the preparation of hydrogels, PEG is usually crosslinked by gamma-irradiation or chemical crosslinking by reaction of hydroxyl groups on the ends of PEG[222] or upon previous functionalization with other functional groups[134,219]. PEG-based synthetic hydrogels are amongst the most studied and employed systems for protein and cell delivery in regenerative medicine because of their good and tailorable mechanical properties, high biocompatibility, and low immunogenic profile, furthermore PEG is commercially available and FDA approved for several applications and it can be easily functionalized incorporating the desired functionalities[223]. Photocrosslinked diacrylate PEG (PEGDA) hydrogels have shown interesting properties. In the study by Nuttelman et al., MSCs were encapsulated within the hydrogel before photocrosslinking and could survive and differentiate into osteoblasts. In particular, cells expressed markers of osteoblastic differentiation such as osteonectin, osteopontin, and alkaline

phosphatase and a mineralization of hydrogels was observed using von Kossa staining[224].

PEG, notwithstanding its high hydrophilicity, is recognized as a biologically inert polymer, consequently both protein and cell adhesion can be fairly limited in PEG-based hydrogels. A frequently employed strategy to improve cell attachment, proliferation and, potentially, differentiation on these substrates consists in the functionalization of PEG hydrogels with RGD motif-containing peptides[136]. Several studies have shown that the incorporation of RGD adhesives peptides increased osteoblast and MSCs attachment, survival, proliferation and differentiation[177], furthermore the mineralization of hydrogels [202],[177]and the production of bone tissue marker proteins were ameliorated[225].

The tri-block copolymer commercially known as Pluronic® F127, made of amphiphilic copolymers polyethylene oxide (PEO) and polypropylene oxide (PPO), $(PEO)_{99}$-$(PPO)_{69}$-$(PEO)_{99}$ can form synthetic hydrogels. Pluronic® is characterized by a thermoreversible gelation: it is liquid at 4°C and forms gel within 5 minutes at 37°C[226]. In addition it has favorable properties such as biocompatibility, non-cytotoxicity and biodegradability[192]. The study conducted by Diniz *et al.* showed that this hydrogel allows adhesion, survival and proliferation of human BMSCs and dental pulp stem cells seeded within the hydrogel. In addition, the authors have shown that when cellularized hydrogels are cultivated in an osteogenic medium, cells express osteogenic differentiation markers and deposit mineralized bone ECM, this making Pluronic a good candidate for BTE[192]. Despite this, the biostability properties and the too rapid degradation in aqueous environment of this hydrogels still limit their use as cellularized scaffolds *in vivo*[192,227].

A valuable alternative are methoxy-PEG-*b*-polycaprolactone (MPEG-PCL) block copolymers that have thermoresponsive properties similar to Pluronic®, low degradation rates and suitable mechanical properties and in the last few years have been successfully tested for *in vivo* osteogenic potential in combination with MSCs from different origins[228,229].

3.3.2.2. Polyphosphoesters-derived hydrogels

Polyphosphoesters are a class of phosphorus-containing polymers featuring repeating phosphoester bonds in their backbone. Polyphosphoester-based hydrogels, by choosing the appropriate starting monomers, (macro)crosslinkers and initiators, can be synthetized by photoinitiated free radical co-polymerization in mild physiological-like conditions and combined with other polymers such as PEG[230]. In addition to their biocompatibility, anyway still dependent on the choice of the building blocks, an

interesting property of polyphosphoester hydrogels is related to the degradation products, in fact the hydrolysis of phosphate linkages produces phosphate, alcohols and diols, with low cytotoxicity[231],[182]. In addition phosphate reacts with the calcium ions present in the surrounding environment producing calcium phosphate thus promoting auto-calcification which may further stimulate cells toward bone ECM deposition[232].

It has been shown that MSCs seeded in polyphosphoester-based hydrogels and incubated in an osteogenic medium survived and led to mineralization of the hydrogel after 3 weeks of culture[231]. The major limit of these hydrogels is a too rapid weight loss over time in culture due to the presence of numerous cleavage sites available for enzymatic biodegradation, leading to a degradation profile that, if not well tuned by optimizing the chemistry of the polymer, may be too fast for the occurrence of an adequate bone regeneration[232].

3.3.2.3. Peptide-derived hydrogels

Synthetic peptide-derived hydrogels are formed by relatively short (around 15-20 residues) amino acid sequences (i.e., self-complementary peptides and peptide amphiphiles) capable of self-assembling into hydrogel networks by physical crosslinking induced by ionic force, pH or temperature changes[219]. These hydrogels show biocompatible, biodegradable and generally non-immunogenic properties, moreover their nanofibrous network organization mimics the natural ECM fibrillar structure[219]. An *in vitro* study of BMSCs seeded within a commercial peptide hydrogel (RADA16®) grown in an osteogenic medium has shown encouraging results for BTE. Indeed, progenitor cells in the hydrogel differentiated into mature osteoblasts and a high and increasing activity of alkaline phosphatase and osteocalcin contents were observed after 2, 3 and 4 weeks of maturation[233]. For *in vivo* use in BTE, the mechanical properties of these hydrogels appear to be low, but some possibilities have proposed to overcome this limitation[234,235], especially based on chemical crosslinking, however it must be ensured that these modifications do not affect the good cytocompatibility and the osteoinduction/osteogenic properties of these hydrogels[235].

Tableau 3 : Matériaux à base d'hydrogels courraments utilisés en ingénierie tissulaire osseuse

Hydrogel material	Origin	Relevant properties	Limitations	Ref.	Relevant studies in BTE
Collagen	Mammals (natural)	Main protein of bone ECM Injectability Cytocompatibility Bioactivity FDA approved	Limited mechanical properties	[14,115,160,194]	*In vitro:* Collagen + BMSCs/Umbilical Cord MSCs: osteogenic differentiation and mineralization[236]
Alginate	Marine algae (natural)	Cytocompatibility Easy chemical modification Tunable properties	Must be pre-formed to be injectable Low cell adhesion; limited mechanical properties	[160,161,237]	*In vitro, in vivo:* RGD grafted alginate + endothelial cells:mineralized ECM deposition[211] *In vivo*: alginate + demineralised bone ECM: ectopic bone mineralized ECM deposition + vascularization[238]
Chitosan	Exoskeletons of crustaceans (natural)	Antibacterial activity Biocompatibility Easy chemical modification Tunable properties	Limited mechanical properties	[210,213]	*In vivo*: chitosan + BMSCs and hydroxyapatite: calvarial bone repair[239]
Pullulan	Fermentation of starch by a fungus (natural)	Cytocompatibility FDA approved	Low cell adhesion	[217,218]	*In vivo:* cholesterol-bearing pullulan + BMP-2: new bone formation in calvarial defects[240]
PEG/PEO	Synthetic polymers	Easy chemical modification Tunable properties Biocompatibility FDA approved	Low cell and protein adhesion; low degradation rate	[182,223,241,242]	*In* vitro: inkjet bioprinted photopolymerized PEG + RGD + BMSC: osteoblast differentiation and scaffold mineralization[177,202] *In vivo:* thermosensitive PEG-b-polycaprolactone copolymers + hDPSCs: osteoblast differentiation and mineralized ECM deposition[228]
PPE	Synthetic polymers	Auto-calcification promotion by degradation products Tunable properties	Fast degradation rate	[182,230,232,243]	*In vitro:* photocrosslinked PPE+ BMSCs: cell survival and deposition of mineralized ECM[231]

Peptides	Amino acid sequences (synthetic)	Cytocompatibility Non-immunogenicity Bioactivity Tunable properties Physical crosslinking induced by ionic force, pH or temperature	Low mechanical properties	[219,234,235]	*In vitro:* RADA16® peptide hydrogel + BMSCs: osteoblast differentiation and mineralized bone ECM deposition[233] *In vivo:* PuraMatrix™ hydrogel + PEEK cage: bone regeneration in bone defects[244,245]

Table 3 abbreviations. ECM: Extra Cellular Matrix; FDA: Food and Drug Administration; MSCs: Mesenchymal Stem Cells; BMSCs: Bone Mesenchymal Stem Cells; PEG: polyethylenglycol; RGD: natural cell binding ligand arginine-glycine-aspartate; BMP: Bone Morphogenetic Protein; hDPSCs: human Dental Pulp Stem Cells; PPE: Polyphosphoester; PEEK: Polyetheretherketone

3.3.3. Multicomponent hydrogels

As we have seen the existing hydrogels for BTE need to compromise between good biological properties (cell attachment, proliferation and differentiation) and good mechanical properties (mechanical resistance to environmental stresses and controlled degradation). Combining different materials is a strategy that may permit to obtain simultaneously biological activity and mechanical support. As mentioned previously, natural hydrogels synthetized from natural polymers such as proteins usually have structures and biological properties which actively regulate cellular responses, offer favorable interactions with the surrounding ECM and promote osteogenesis. Oppositely, synthetic hydrogels are often associated with higher mechanical properties and biostability. Consequently, the combination of the characteristics of the synthetic and natural polymers to design hybrid hydrogels is envisaged as a promising approach for the creation of bioactive scaffolds for BTE[219]. One of the most employed approaches involves the combination of natural polymers with PEG. Coupling a biological molecule to PEG usually contributes to improve the biological activity of the synthetic polymer and confers to otherwise biologically inert PEG hydrogels, cell/protein adhesion properties[222]. Several studies combined PEG with naturally derived ECM components such as collagen[246] or hyaluronic acid[247] and showed enhanced biological properties with high (encapsulated) stem cells viability *in vitro* and *in vivo* associated with enhanced mechanical properties.

Pullulan has been recently blended with dextran and sodium carbonate as porogen reagent to form hydrogels with interconnected pores of 200 µm that were seeded with MSCs. These systems demonstrated superior osteogenesis *in vivo* in a rat model of large bone defect with the hydrogel that was rapidly resorbed and substituted by a dense mineralized bone tissue forming from the edges of the defect. SMCs promoted both bone formation and vascularization but it was not clear if they exerted a paracrine effect, a direct bone tissue deposition activity through their differentiation into mature osteoblasts, or a combination of the two processes, since their number was extremely decreased 30 days after implantation[248].

Another example is PNIPAAM, a temperature-responsive polymer which have the abilities to form hydrogel when heated at 32°C in water. This synthetic hydrogel has been shown to be a good candidate for the encapsulation of bone cells[249] but its use is limited because of its poor biocompatibility and non-biodegradability[250]. To overcome these limitations many researches modified this hydrogel with natural compounds including collagen[251], chitosan[252], hyaluronic acid[253] or RGD peptides[254]. Liao

et al. have shown that hyaluronic acid-chitosan-PNIPAM hydrogels can promote MSCs proliferation and osteogenic differentiation and secretion of mineralized ECM after culture in an osteogenic environment. Also *in vivo* grafting of the injectable hydrogel-MSCs complex demonstrated ectopic bone formation and total biodegradation of the material without toxic reaction to animals[255].

3.3.4. Composite Hydrogel

Composite hydrogels aim to combine natural or synthetic hydrogels with bioactive phases, degradable polymeric structures and/or bio-ceramics to enhance the mechanical and biological properties of each compound to produce a relevant environment for BTE.

To increase osteoinductive, osteoconductive and mechanical properties of hydrogels one strategy consists in loading them with usually micro/nano-sized mineral phase-like ceramics to promote tissue formation while providing higher initial mechanical properties to bear the solicitations occurring in the bone environment[207]. Thus, new composite matrices combining polymers and calcium phosphates have been developed to mimic as closely as possible the bone matrix, a mixture of organic and inorganic components. Elements based on calcium phosphates provide mechanical properties and osteoconductivity and the polymer component, collagen or chitosan, alginate, improves the biocompatibility and biodegradability of the biomaterial[136]. Three of the most commonly used mineral supplemented matrices in BTE are calcium phosphate ceramics, tricalcium phosphates and hydroxyapatite. These mineral compounds show adequate biocompatibility and suitable osteoconduction and osseointegration properties[256]. In one of these biomimetic approaches, a composite hydrogel of type I collagen and hydroxyapatite could enhance osteoblast differentiation[257] and accelerate osteogenesis[258]. Alginate hydrogels blended with hydroxyapatite were reported to support the adhesion and proliferation of osteosarcoma MG-63 human cell line. The system showed at the same time adequate structural and physical-chemical properties for being used as scaffolds in BTE strategies but it is not injectable[259].

I must be noted that, in general, the mechanisms of interaction between hydrogel networks and the supplemented inorganic particles still need to be elucidated in depth. Data about *in vivo* applications of these systems are still limited, making necessary further comprehensive studies on the long-term performances, cytotoxicity, biocompatibility, biodegradability and osteogenic activity of such composite hydrogels under *in vivo* conditions to confirm the promising properties of this class of materials for BTE[260].

Cellularised hydrogels can also be used as fillers of degradable porous polymeric structures serving as bone grafts that temporarily bear loads. Heo *et al.*, for example, have recently combined 3D-printed polymeric porous microstructures with photo-curable gelatin hydrogels laden with adipose-derived stem cells (hASCs) that demonstrated osteogenic capability *in vitro*[261].

3.4. Choosing and preparing cells for BTE

3.4.1. The different cell types used for BTE

Regenerating bone in areas where no/few suitable progenitor cells are available to differentiate and synthesize and deposit an osteoid matrix requires the input of exogenous cells which, associated with an appropriate scaffold and other factors, will differentiate into functional osteoblasts, the primary actors of bone formation. The choice of the source of osteoblast progenitors and the procedure used to isolate, amplify and prepare them before seeding the 3D scaffold and grafting the construct in the host site have significant consequences on the efficiency of the BTE product. This choice must consider several parameters and will usually result from a compromise between advantages and drawbacks.

3.4.1.1. Adult mesenchymal stem cells

Mesenchymal stromal cells (MSCs) are the most widely used stem cells for BTE applications. This statement is supported by the large predominance of publications where the keywords “bone tissue engineering” are associated with the word “MSCs” (5539 articles and 700 reviews found in Pubmed[262]) over association with other cell types (492 articles and 181 reviews for embryonic stem cells and191 articles and 66 reviews for induced pluripotent stem cells). Moreover, there are currently 24 clinical trials ongoing for the treatment of bone fractures that use MSCs whereas none is so far reported using embryonic stem (ES) or induced pluripotent stem cells (iPSCs)[263].

MSCs used for BTE have been obtained from several tissues and organs, including bone marrow[129], adipose tissue[264,265], amniotic fluid[266], dental pulp, or Whartons’s jelly[267]. A specific and selective cell surface marker for the MSC has yet to be determined, but these cells are typically identified by their expression of CD90, CD105, CD73, and CD146, and absence of CD45, CD34, CD14, CD11b, CD79a, CD19, HLA-DR. Without clear markers for cell sorting, the International Society for Cellular Therapy has proposed a set of basic requirements for a cell to be classified as a MSC. MSCs are defined as a plastic culture adhesive cell with the ability to generate a colony-forming unit and differentiate into bone, cartilage, and adipose tissues[268].

MSC from different origins show important similarities in their transcriptome profile, but significant differences in the expression of a subset of genes have been observed[269,270]. These differences in gene expression have not been correlated with functional differences, but clearly demonstrate that MSC identity depends on their origin, and suggest that the origin may explain the phenotypic differences observed upon *in vitro* and *in vivo* manipulation of these cells. Several studies have reported differences in proliferation and differentiation capacity of MSC according to their tissue origin, when they are grown in identical conditions *in vitro*. Concerning proliferation capacity, no consensus emerges, some studies reporting for instance a higher proliferation rate for bone marrow MSCs (BMSC) over adipose derived stem cells (hASC)[271], others showing the opposite behavior[272]. The differentiation potential under identical culture conditions yields a clearer picture, with a significantly higher osteoblastic differentiation potential for BMSC over hASC for instance, reported by many studies under different micro-environments[271,272]. The more recently characterized dental pulp-derived stem cells exhibit superior osteogenic properties as compared to the two previously mentionedMSC subsets and this increased capacity to differentiate into osteoblasts has been correlated with improved bone formation *in vivo*[273]. The capacity of MSCs to elicit endothelial cell differentiation is limited regardless of MSC origin, however BMSCs seem to have a higher potential[274].

At this point the choice between these different sources of MSCs may be determined not only according to the differentiation capacities but to the easiness of cell harvesting and amount of cells that can be collected. In this respect, adipose tissue should be ranked first, for its easy access and high proportion of MSCs within the stromal vascular fraction, obtained by digesting the fat and concentrating the remaining cells. Isolation of cells from dental pulp, although the proportion of MSCs is very high, requires wisdom tooth extraction, which is not really applicable to most patients. Other sources of stem cells are also compromised by the difficulty to obtain them or by their low quantity, and are therefore likely to remain models used in fundamental research for the studies of stem cell differentiation and repair capacity, without real therapeutic applications. For instance, MSCs from cortical bone show a very high osteoblast differentiation capacity and *in vivo* osteogenic potential[275], but the difficulty to obtain them rules them out any therapeutic perspective. Likewise, periosteal stem cells are perhaps the most relevant to bone regeneration since they are the primary source of cells that heal the fracture[276], but these cells are unlikely to play a significant role in therapeutic strategies since periosteal stripping could negatively impact normal bone homeostasis and cause donor site morbidity. These cells are also difficult to access and available in low quantity.

Two major drawbacks of MSCs are heterogeneity[277] and donor-dependent variability[278]. A promising route to improve the efficiency of MSCs is to select and isolate specific sub-populations, using cell sorting based on specific markers[279,280]. For instance, a recent study by König et al showed the superior bone forming capacity of CD146+ pericytes from fat tissue[281]. However, this selection procedure is likely to reduce the amount of cells produced from the biopsies and available for clinical applications.

In addition to their osteoblast differentiation capacity, MSCs play an important role in regulating inflammation and have a trophic function in stimulating tissue regeneration[282]. The immunomodulatory role of MSCs plays a critical role both in normal healing and in therapeutic approaches. Immunomodulation by the MSCs is accomplished by secretion of immunosuppressive and anti-inflammatory cytokines, such as interleukin-10, nitric oxide and prostaglandins. MSCs can also regulate T-cells in an antigen-independent manner through the suppression of the primary and secondary T-cell responses by inhibiting cell proliferation. MSCs also promote a local healing response by stimulating proliferation and differentiation of resident stem cell populations, reducing fibrosis, and inhibiting adverse apoptosis. MSCs secrete several cytokines such as TGF-β, stem cell factor (SCF), insulin-like growth factor (IGF), epidermal growth factor (EGF), and granulocyte and macrophage colony stimulating factors (G/M-CSF). Taken together, the immunosuppressive and trophic capabilities of MSCs are powerful and may play an important part in the tissue regeneration process. Of particular interest is how MSCs appear to have a lasting therapeutic effect despite a transient persistence after engraftment. Indeed, a major limitation of MSCs is their short lifespan after implantation. In many studies where MSC number is monitored, more than 90% of the grafted cells die within 14 days[283,284]. Anoikis has been shown to be a major cause of cell death when cells do not adhere to the matrix to which they have been associated[285]. Ischemia is another major cause for the death of grafted cells[286], and the association of oxygen carriers to BTE constructs has been shown to improve survival and the bone regeneration capacity of the cells[287,288]. The availability of glucose is another key parameter, and combinations of low glucose and low oxygen result in severe cell loss[289,290].

3.4.1.1.1. Influence of the procedures used for isolation, maintenance and conditioning of MSCs

The production of cells from human tissues involves several steps. The isolation procedure involves mechanical action, sometimes enzymatic digestion, and always an

abrupt change of the physical and biochemical environment. Indeed, cells are suddenly transferred from a soft and relatively hypoxic micro-environment in their tissue of origin, into a solution under 21% oxygen during the time of dissociation, and then transferred again onto a very stiff plastic culture dish, in a special culture medium. Modification of matrix stiffness[291–293] and oxygen concentration[294] have been extensively shown to critically affect cell phenotype. The transient exposure of cells to a very stiff substrate (such as plastic) has been shown to favor osteoblast differentiation, that was maintained even after cells were transferred to a softer substrate, such as 3D matrices for implantation[291].Following this stressful treatment, they will be usually grown for several days, and will often have to undergo a few passages, involving cycles of trypsinization and re-plating. Although the consequences of each of these steps have not been examined thoroughly, some studies report significant consequences of the procedures used to isolate and grow cells prior to their embedding in the host 3D matrix which will be used for engraftment. The protocols used for harvesting MSCs from bone has been shown to affect their capacity of differentiation towards osteoblasts[295]. Plating and passages have been shown to alter the pattern of expression of several surface markers[296] and more generally affects their transcriptome[297]. Such modifications seem to affect the capacity of MSCs, once seeded into a 3D matrix and implanted in host tissues, to differentiate into osteoblasts and, most importantly, to efficiently produce a bone matrix[298]. Interestingly, treatment of cells with melatonin during this expansion period, has been proposed to preserve their differentiation capacity[299]. Given the loss of performances of MSCs upon long-term culture, it is advisable to reduce the number of passages (usually MSCs undergo less than 5 passages before engraftment), although a compromise must be found between a large number of cells, requiring prolonged amplification, and maintenance of therapeutic efficiency of cells. A recently proposed alternative to expansion in 2D culture of MSCs is their growth as spheroids. MSCs spontaneously associate to form these structures when they are grown on a low-adhesive substrate. These structures evolve within hours from a loose aggregate towards a compact sphere. Interactions between cells are much more abundant in these structures than in 2D cultures, and cells are exposed in different environments according to their position within the spheroid. Several studies have shown that the phenotype of MSCs in spheroids differs in many aspects from the one of cells grown in 2D: stemness, differentiation capacities, immune-modulatory and anti-inflammatory effects are enhanced in 3D aggregates[300]. In terms bone regeneration potential, spheroids have been shown to favor osteoblast differentiation, especially in the absence of osteoinductive factors in the culture medium. They also exhibit increased secretion of VEGF, potentially favoring the vascularization of newly formed bone[301].

3.4.1.2. Human ES cells and iPSCs

The capacity of human embryonic stem (ES) cells to produce bone matrix has been tested with different protocols and scaffolds. Two strategies have been explored, the direct differentiation of ES cells towards osteoblasts, or the prior differentiation towards MSCs, yielding ESC-derived MSCs, which were subsequently driven towards the osteoblastic lineage[302]. Direct differentiation of ES cells to osteoblasts was achieved using osteoinductive scaffolds and the classical osteogenic culture medium. Studies have shown a higher proliferation rate with ES cells than with MSCs, favoring the colonization of the scaffold[284]. However, ES cells show notable tumorigenic properties: they are characterized by high telomerase activity (which leads to potentially infinite proliferation) and are known to form teratomas[303,304]. Nonetheless, ES cells handling is surrounded by several ethical issues due to their embryonic provenance, thus making improbable their use in bone defects treatment, at least in the near future.

The use of induced pluripotent stem cells (iPSCs) for BTE has emerged after the initial description of the reprogramming of different human somatic adult cells[305]. In order to avoid tumorigenic potential of these cells, they have to be pre-differentiated towards the mesoderm lineage before being implanted. This can be achieved either via the formation of intermediary embryoid bodies or directly from isolated cells[306]. In all cases, different osteogenic media were used, either supplemented with β-glycerol phosphate, ascorbic acid and dexamethasone, or with growth factors such as TGF-β, insulin growth factor-1 (IGF-1), basic fibroblast growth factor (FGF-β), or BMP-2, and they showed to enhance the osteogenic capability of iPSCs. The types of scaffolds used to grow, differentiate and implant iPSCs were not different from those used for MSC, including natural or synthetic polymers, combinations of both, sometimes combined with an osteo-conductive hydroxyapatite component[307]. An important issue, as for MSCs, was to compare the osteogenic capacity of iPSCs according to their tissue of origin. A few studies report improved osteogenic properties of iPSCs derived from bone marrow stromal cells as compared with other sources, both *in vitro* and *in vivo*[308]. Although cells generated from bone marrow exhibited a higher osteogenic potential, all sources were used successfully to produce bone tissue. Several studies have also shown that bone formation could be obtained either by the direct use of iPSCs or via prior formation of embryoid bodies[309]. These cell sources seem promising but it remains to be checked that implanted cells do not form teratomas on the long term.

The great advantage of using iPSCs or ES cells compared to MSC is that these pluripotent cells can be grown for a considerable period before differentiation is induced. They maintain their pluripotent property during this amplification step, and only

afterwards can they be induced towards the osteoblastic differentiation pathway[310,311]. Besides, it is possible to generate different specialized cell types from a single source of iPSCs, enabling the design of more complex TEPs. For instance, Jeon *et al* have shown that co-implanting osteoblasts and osteoclasts obtained from iPSCs in a HA-coated poly(lactic-co-glycolic acid)/poly(L-lactic acid) scaffolds matrix elicited enhanced ectopic bone formation[312].

3.4.2. Creating an environment to favor angiogenesis

The efficiency of the tissue engineering products largely depends on their capacity to be rapidly colonized by blood vessels to ensure oxygen and nutrient supply to the embedded cells. Consequently, several strategies have been developed to favor angiogenesis around and within the implanted tissue engineering constructs. Two types of factors determine the efficiency of blood vessel colonization of the scaffold: (i) the macroporosity, which must be sufficient to enable the progression of new blood vessels and, as described above, largely depends on the structure of the hydrogel; and (ii) the angiogenic potential of the scaffold itself. This potential can be enhanced by the release of angiogenic growth factors by the scaffold (or by their secretion by the embedded cells).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor[313]. This protein is produced by several cell types including MSCs[314], but the secretion of VEGF by these cells largely depends on the amount of grafted cells, culture conditions[315–317], tissue origin [274]and other environmental factors, limiting its potential role in neo-angiogenesis. A few studies have addressed the possibility to deliver VEGF at the site of implantation of biomaterials to stimulate angiogenesis from the host tissue, either alone or in combination with other growth factors such as BMP-2[318]. The results obtained with these strategies show a limited positive effect of VEGF on the amount and quality of newly formed bone. This modest input by VEGF may be due to the fact that VEGF efficiency is dependent on several parameters such as spatial distribution, association with matrix proteins and time-dependent availability[319]. Consequently, strategies aimed at controlling the release of VEGF have shown improved efficiency[320] but they should be further developed. Some of these limitations may be also overcome by the use of transgene-mediated VEGF production[318], but this requires the prior infection or transfection of cells before their association with the scaffold, a procedure that raises additional safety and regulatory issues for therapeutic applications.

An alternative to the use of growth factors to promote angiogenesis is to incorporate endothelial cells or endothelial cell progenitors in the hydrogels, eventually in combination with other cell types such as MSCs or with growth factors. Two types of

endothelial cell sources are essentially used: mature endothelial cells generally isolated from umbilical cord vein (HUVEC) or endothelial progenitor cells (ECP) isolated from blood. Because of the possibility to isolate EPCs from the patient to perform autografts, EPCs represent a more attractive source of cells and are therefore preferred in most of recent studies. EPCs alone, when associated to different scaffolds and implanted in different tissues, have been shown to trigger angiogenesis[267,321]. However, they do not promote bone formation by themselves. Co-culture followed by co-implantation, or direct co-implantation of MSCs and EPCs have been shown to result in enhanced osteogenesis, as compared with MSCs alone, but not to significant differences in angiogenic properties of EPCs alone. These studies support the notion that MSCs do not enhance the capacity of EPCs to make new blood vessels. Instead, they show that EPCs potentiate the capacity of MSCs to elicit bone formation[322]. Noteworthy, some studies have shown that using differentiated osteoblasts instead of un-differentiated MSCs in co-culture with endothelial progenitors favored blood vessel formation[323]. Moreover, osteoblasts have been shown to stimulate angiogenesis by the host tissue[324,325]. It is however difficult from the available data to determine whether bone formation is increased because more blood vessels irrigate the scaffold and favor cell viability and function, or whether this synergy results from early, direct cell-cell interactions between MSCs and EPCs. Several in vitro studies have shown direct interactions between these two types of cells, both in 2D[326] and in 3D cultures[327], and stimulation of MSC differentiation towards the osteoblastic phenotype. Thus, contact between both cell types during the pre-culture period or within the scaffold upon implantation is probably mandatory to promote increased bone formation.In this context, a new approach consists in the generation of scaffolds integrating osteogenic and angiogenic niches in the hydrogel structure. Photolithography was used by Kazemzadeh-Narbat *et al.* to control the photocrosslinkable hydrogel stiffness and the patterned distribution of ECs, MSCs and preosteoblasts, allowing to obtain, *in vitro*, mineralized regions surrounded by organized vasculature[328]. The interesting results reported in this proof-of-concept study, performed on a construct with planar geometry, are very promising for the translation of this approach to more complex shapes and for the possible application for treatment of bone defects.

3.4.3. Pre-conditioning cells before engraftment

Cell fate is determined by the combination of several biophysical and biochemical parameters[329]. Usually, cells are amplified on tissue plastic dishes in a basal, non-osteogenic medium. However, after embedding into the implantable 3D scaffold, cells can be submitted to very different micro-environments, which significantly affect their *in*

vivo fate[330]. The parameters of this environment include the following options: incorporation of growth factors, using either the scaffold itself as delivery system, or using intermediate carriers such as nanoparticles[331]; the incorporation of hydroxyapatite particles with different physical characteristics; pre-differentiation, or not, before implantation; perfusion of the cellularized scaffolds in bioreactors; control of oxygen concentration. Combinations of the above-mentioned parameters offer the possibility to create an infinity of different micro-environments.

Whether or not, cells should be pre-differentiated before their implantation is an important issue. Pre-differentiating MSCs towards osteoblastic lineage has been shown to improve their bone formation potential, but also their survival after engraftment[332]. However, some studies have shown that this pre-differentiation step reduces their intrinsic angiogenic properties[333]. When grown as spheroids, MSCs have an enhanced capacity to differentiate into osteoblasts without osteogenic culture medium, however the capacity of these structures to elicit bone regeneration is limited[334]. Although it may improve cell efficiency, pre-differentiation implies prolonged culture time and hence increased risks of contamination and mutations, and higher costs, all parameters which are not desirable for therapeutic applications. Therefore, association with osteogenic growth factors, culture in hypoxic conditions or short-term mechanical stimulation are promising alternatives which are presented herein below.

3.4.3.1. Growth factors

As described above, prominent growth factors involved in bone formation and repair are TGF-β, BMPs, FGFs, EGF, IGFs, PDGF. Growth factors of the TGF-β super family such as BMPs induced primary signal to upregulate mineral-depositing osteoblasts diferenciation from pluripotent cells wich are present within scaffold or in the host tissue[335]. Moreover, BMP-2 and BMP-7 are approved by FDA to be used in treatements of spinal fusions and long-bone fractures in association with a collagen carrier[336]. The main limitations of the use of BMP2 are the use of supraphysiological doses which may lead to complications such as immune reactions, formation of ectopic bone tissue and oedemas[337,338]. To overcome these limitations one strategy is to use hydrogels to sequester the growth factors and slowly release them in the site of morbidity for the upregulation of suitable cellular activity. Among the various strategies proposed there is for example the functionalization of hydrogels with heparin because of its affinity for BMP-2[339,340]. Such systems aim at preventing burst release and favor sustained release of BMP-2 to promote mineral deposition within the injured site[136]. Thermosensitive hydrogels are also good candidates because they allow to incorporate

BMP-2 by simply mixing it at the liquid polymer phase before gelation *in situ* at physiological temperature. Seo *et al.* have shown the ability of injectable thermosensitive polymeric nanoparticle hydrogels to efficiently carry and release BMP-2 in a sustained and controlled fashion both *in vitro* and *in vivo*. They also showed that, *in vivo,* hydrogel-carried BMP-2 was able to promote new bone generation and infiltration of bone/progenitor cells from the surrounding tissues within the hydrogel without inflammatory responses upon each injection[336]. Although BMP-2 by itself has always shown strong osteogenic potency, there is still debate about its effects on exogenous grafted cells in tissue engineering products. In fact, BMP-2 remains the most widely studied factor, with a significant action on the survival and differentiation of MSCs[341], however, dependently on the tissue of origin of MSC, there are discrepant results concerning the effects of BMP-2. For instance, the efficiency of BMP-2 on hASCs is controversial, some studies reporting a significant osteogenic effect[342–344], others showing no effect at all[345]. The action of BMP-2 is more consensual on BMSCs, with positive effects on cell survival *in vivo*[335], and on bone formation. BMP-2 has also been shown to stimulate bone formation by human ES cells[346] and iPSCs[307].

As an alternative to BMPs, other growth factors within the above-mentioned list, individually or in combinations, have been shown to have positive effects on MSC survival or MSC-mediated bone formation, such as EGF[347], TGF-β and FGF[348] and PDGF[349]. Some bioactive small molecular weight compounds have also been shown to favor stem-cell mediated bone formation. For example, some studies report an osteogenic effect of icariin[350] or simvastatin[351] loaded inside cellularized BTE scaffolds.

A more recently identified family of molecules that may be used to induce MSC differentiation towards osteoblasts is microRNAs (miRNA)[352]. miRNAs are short single strand non-coding molecules of RNA (between 18 and 24 nucleotides long) acting in the cytoplasm as gene inhibitors and used by cells to regulate the expression of many genes by RNA interference[353]. Several gene delivery techniques and approaches can be employed to ferry miRNA to cells and this approach appears to be a promising tool to stimulate *in vivo* bone formation in the presence of MSCs[354,355]. Enhancement of osteogenic or angiogenic properties of MSCs has been achieved by introducing in these cells plasmids or adenoviruses encoding for the expression of growth factors such as BMP-2 or angiopoietin[356], respectively. Although these tools have been proved to be usually more efficient that soluble factors[357] because they allow a continuous, long-term delivery of the active proteins, therapeutic applications are likely to be hindered by

risks associated with the introduction of exogenous nucleic acid sequences in grafted cells. Transient transfection of siRNAs may be more acceptable and has been shown to improve MSC performances[358].

3.4.3.2. Oxygen control

Since stem cells are amplified in 21% O_2 atmosphere before being grafted into a tissue where O_2 concentration drops to 3-5%, a tempting strategy is to pre-condition them so that they can adapt their metabolism to improve their survival and performances once implanted in the harsh tissue environment, at least until neo-vascularization restores normal nutrient and oxygen supply[359]. Pre-conditioning of MSCs by growing them in hypoxic conditions has shown some benefits on MSC survival and on some of their physiological properties[360], but to our knowledge, these strategies have not been explored in the context of *in vivo* bone formation or bone defect repair. An alternative strategy to help cell surviving the *in vivo* hypoxic environment is to provide them with an extra oxygen store using synthetic oxygen carriers, which can slowly release oxygen transiently until vascularization is restored[287,288]. This strategy has been shown to enhance bone formation by implanted MSCs.

3.4.3.3. Mechanical stimulation

Whereas cells are commonly grown *in vitro* on 2D platforms and under static conditions, a number of studies have shown that growing MSCs in 3D matrices, and in addition under mechanical stimulation, considerably modifies their phenotype as compared to classical 2D culture. Different types of mechanical stimuli can be applied to the cell-containing scaffold. It is possible to apply compressive or tensile loads with defined strength and frequency to the scaffold, resulting in the deformation of its structure and modification of cell adhesion. The effect of compressive loads has been shown to be positive on the *in vitro* differentiation of MSCs towards osteoblasts[361], but its impact on *in vivo* performances remains unexplored. Another type of stimulation consists in perfusion of the cellularized scaffolds with culture medium in bioreactors, controlling flow rate and pressure. Submitting embedded cells to fluid flow has been shown to enhance their osteoblastic differentiation and bone formation[362]. Even a short session of fluid perfusion has been shown to increase osteoblast maturation[363]. Such perfusion systems have also been shown to be effective to produce MSCs, and subsequently osteoblasts, from human ES cells and iPSCs[364]. These mechanical treatments are however difficult to reproduce in all laboratories as the fine control of all the parameters, which considerably influence the effect on cell phenotype, heavily depends on the type

of equipment used to generate and control the flow. In addition, their benefit for the *in vivo* efficiency of MSCs is still not convincingly documented[365].

3.4.3.4. Contribution of exogenous versus host cells in tissue regeneration

Many combinations of scaffold/stem cells/growth factors have proved to be osteogenic and to promote bone defect repair. The benefit of the presence of stem cells within the implanted scaffold has been also demonstrated in a large number of studies. However, one key question is how grafted cells contribute to tissue regeneration. In terms of experimental data, two question can be formulated: “what percentage of the initially implanted cells are still present within the newly formed tissue at the end of the experiment (when new tissue has been formed), and what is their differentiation status?” and “what is the percentage of exogenous versus host cells which participate in the construction of the new tissue? These data are usually not available from the published work. In some instances however, careful quantification of human stem cells and of host mouse cells has been achieved in the biopsies, and provides interesting clues. In a recent study, Nuschke *et al*. have analyzed the effect of EGF, tethered to tri-calcium phosphate particles and embedded in a collagen matrix, together with primary BMSCs[347]. They report a positive effect of EGF on the survival of exogenous cells. But this study also shows that the proportion of exogenous cells is very low (less than 10%), as compared to host cells, and decreases over time. These observations suggest that new tissue is not built primarily by exogenous MSCs, but essentially by endogenous cells. These observations support an indirect, paracrine effect of MSCs. In contrast, some studies provide convincing evidence for the direct involvement of the human grafted cells, supported by the deposition of human collagen[366]. Two major differences can be noted between both models: in the first study, BMSCs were directly implanted without any pre-culture, whereas in the latter, the construct containing ASCs was incubated for 14 days in an osteogenic medium. Both MSC types were seeded on a β-TCP scaffold. Although it is difficult to draw conclusions from this unique comparison, one can speculate that the fate of implanted cells is likely to be affected by the pre-conditioning, in particular submission of the tissue engineering product to a pre-culture or not, and the conditions used for this pre-culture. Another study shows that iPSCs, pre-differentiated into osteoblasts, efficiently promote bone formation and can be quantitatively found after several weeks[367]. A study by Binder et al compared MSCs implanted after culture either in basal or in osteogenic medium, and observed a very significantly higher survival when cells were pre-cultured in osteogenic medium. In parallel, bone formation is also increased in these conditions[368].

3.5. Conclusions and future direction

From the current review, some conclusions can be drawn but many questions are still pending.

Raising the issue of an ideal “carrier” or “scaffold” for bone repair cells seems at the moment a non-sense, because in fact the definition itself of such a perfect material is not univocal. According to the type of bone defect, (anatomical location, size, shape) and to the quality of the surrounding tissues, which depends on the cause of the bone damage but also on several of the patient’s physiological parameters, different materials could be the best choice. Many types of hydrogels have been shown to have osteoinductive and osteogenic properties in the presence of cells and growth factors, but their translation to clinical application relies on other parameters such as injectability, biocompatibility, mechanical stability and biodegradation rate. These properties may certainly have to be adapted to the specific therapeutic application and, as mentioned above, patient-dependent parameters should be taken into account. Hydrogels appear as the systems of choice for cell transplantation and many recent studies have pointed out that combinatorial approaches, employing blends of natural and/or synthetic polymers with different properties, are the right way to follow to overcome the limitations of classical hydrogel-forming materials, even if controlling at the same time relevant hydrogel parameters such as mechanical properties, degradability, porosity, biocompatibility and bioactivity is hardly possible. In addition, the incorporation of calcium phosphate particles, mimicking the inorganic phase of bone ECM, has been very often shown to confer improved osteoconduction and osteoinduction to the scaffold but also osteogenic potential to the grafted cells. One of the greatest challenges in hydrogel-based systems for BTE remains the achievement of suitable mechanical properties for the treatment of load-bearing defects. The possibility of designing scaffolds by 3D printing techniques will certainly enable to better control the structural, and hence mechanical and biological properties of the products. However, the success of these new approaches in bone tissue regeneration largely depends on the capacity of the researchers to model the scaffold they want to produce, and hence to be able to establish clear structure-bioactivity relationships. This is a complex challenge, and whereas so far some success has been met at the *in vitro* level, the ability to design structures that will be able to fulfill their function once in the complex *in vivo* environment is still far ahead. Composite systems, combining hydrogels with solid phases (e.g., degradable polymeric structures, bioceramics), are another promising alternative since they can provide synergistic biological activity together with mechanical reinforcement, but their actual *in vivo* potential still need to be explored.

If the choice or design of the best scaffold is not easy, choosing the best cell source and the best way to handle and prepare them is another challenging issue. In addition to pure efficiency criteria, the choice of the cell source should take into account also cell availability, costs associated to cell expansion and pre-conditioning, safety issues and also ethical concerns Ethical issues are essentially related to the use of embryonic stem cells that, despite their many practical advantages and their almost unlimited potential, cannot be considered at the moment the cell type of choice. iPSCs suffer from difficult handling and insufficient proof of safety, but the rapid development of knowledge and techniques on these cells should rapidly overcome these limitations. Considering all the adult stem cell sources, there are only minor differences in cell survival, osteoblastic differentiation capacity and bone forming activity, based solely on the origin of the cells used. These differences can be smoothed by the association with appropriate growth factors, co-embedded with cells and released in a controlled manner by the use of different delivery systems. In conclusion, the choice of the cell source may be governed by practical issues such as availability and costs, but also adapted to the patient's health status and physiological characteristics. For instance bone marrow, adipose tissue, and dental pulp represent three major sources of autologous MSCs, and the choice of the donor tissue may be patient-dependent. iPSCs, which also show a high degree of patient donor-dependency, should probably be tested for their efficiency before engaging into a long-lasting and probably expensive process. In this context, the identification of markers that would predict the potential of different adult cells from various tissues to generate highly efficient iPSCs could help making the best choice.

If different stem cells are eligible as bone repair cells, very little is known about the mechanism by which they contribute to the bone regeneration process.

In vivo cell survival is still poorly investigated, and very few studies have addressed this question in a quantitative manner. Survival clearly seems favored by pre-culture in an osteogenic medium, and this prolonged life time is associated with improved bone formation, suggesting that abundant functional cells are necessary to obtain efficient bone regeneration. The drawback of such procedure is its costs and the risks of genetic alterations upon prolonged culture. The co-grafting of undifferentiated cells combined with the controlled release of osteogenic growth factors is undoubtedly a promising alternative to extensive pre-culture.

A lot of work has yet to be done to characterize the role and the fate of grafted cells. Imaging tools to follow the fate of implanted cells, to localize them and more importantly to quantify them, are available or under rapid development[369]. The interplay between

exogenous and resident cells is another poorly explored question, and the rich secretome of MSCs certainly plays a major role in the recruitment, maturation and organization of the resident cells within the regenerating tissue. A better knowledge of cell behavior upon transplantation will be pivotal in drawing guidelines for the design of hydrogel-based systems with mechanical, structural and biological properties optimized for osteogenesis.

Finally, the issue of a rapid and efficient vascularization of the grafted TEPs remains one of the most challenging. Endothelial cells or their progenitors and angiogenic growth factors have proved to be efficient in eliciting the formation of new blood vessels. But the anastomosis of these newly formed vessels with those of the host, and the quality and function of this neo-vasculature are far from being optimal. In this respect, 3D printing technologies might bring a decisive input, because they allow not only the construction of perfectly controlled scaffold structures (with interconnected pores to allow vessel invasion), but also the printing of endothelial cells progenitors with high resolution, in order to favor the rapid formation of capillaries within the macroscopic bone tissue engineering scaffold. Unfortunately, the use of 3D printing is not compatible with the injectability of the system.

Concluding, multicomponent composite systems appear as the new generation of hydrogel-based systems, where incremental improvements obtained in the past research can be merged synergistically. However, it is quite apparent that combining all the desired properties in “ideal” cellularised scaffolds is a utopia and compromises need to be done in their conception. The future solutions to bone repair challenges might come from the application of complementary technologies and techniques based on the precise control, at different scales, of the organization of osteogenic and angiogenic actors in a single, highly structured scaffold where accurately selected and preconditioned cells can find a suitable physiological-like environment to guide bone tissue regeneration.

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4. A new composite hydrogel combining the biological properties of collagen with the mechanical properties of a supramolecular scaffold for bone tissue engineering.

Mathieu Maisani[1,2], Sophia Ziane[1], Camille Ehret[1], Lucie Levesque[2], Robin Siadous[1], Jean-François Le Meins[3], Pascale Chevalier[2], Philippe Barthélémy[4], Hugo De Oliveira[1], Joëlle Amédée[1], Diego Mantovani[2], Olivier Chassande[1]

[1]Laboratoire BIOTIS, Inserm U1026, Université de Bordeaux, Bordeaux, France

[2]Lab. for Biomaterials & Bioengineering CRC-I, Dept. Min-Met-Materials Engineering & Research Center CHU de Québec, Laval University, Québec City, Canada

[3]Laboratoire de Chimie des Polymères Organiques LCPO (UMR5629)-Ecole Nationale Supérieure de Chimie, de Biologie et de Physique (ENSCBP) Institut Polytechnique de Bordeaux, France

[4]ChemBioPharm, Inserm U1212, Université de Bordeaux, France

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4.1. Résumé

L'ingénierie tissulaire est une alternative prometteuse aux autogreffes, aux allogreffes ou aux biomatériaux pour le traitement des lésions osseuses de tailles critiques. Généralement les produits d'ingénierie tissulaire associent un échafaudage et des cellules et sont implantés ou injectés au sein de la lésion. Les cellules doivent être intégrés au sein de l'échafaudage biocompatible et approprié afin d'offrir un environnement favorable à leur survie et leur différenciation en cellules osseuses. Au cours de nos travaux, nous avons développé un hydrogel composite par l'association d'un hydrogel de collagène de type 1, une protéine de la matrice extracellulaire largement utilisée dans plusieurs applications thérapeutiques, avec un hydrogel synthétique généré à partir d'une molécule amphiphile. Cet hydrogel composite a présenté des propriétés mécaniques et biologiques améliorés par rapport à celles présentées par chacun des hydrogels utilisés individuellement. L'incorporation de l'hydrogel synthétique participe à limitation de l'effet de compaction de l'hydrogel de collagène et a augmenté le module élastique de l'hydrogel. L'hydrogel composite a permis l'adhésion, la survie et la prolifération cellulaire *in vitro* et *in vivo*. De plus, il a favorisé la différenciation des hASCs en l'absence de facteurs ostéogéniques. *In vivo*, les cellules ensemencées au sein de l'hydrogel composite et injectées par voie sous-cutanée chez la souris ont produit un tissu lamellaire et se sont différenciées en ostéoblastes. Cette étude présente ce nouvel hydrogel composite comme une échafaudage prometteur pour une application en ingénierie tissulaire osseuse.

Mots-clés

Os, Ingénierie tissulaire, Hydrogels, Ostéogénique, Biomatériaux, Cellules souches, Échafaudages

Abstract

Tissue engineering is a promising alternative to autografts, allografts or biomaterials to address the treatment of severe and large bone lesions. Classically, tissue engineering products associate a scaffold and cells, and are implanted or injected into the lesion. These cells must be embedded in an appropriate biocompatible scaffold, which offers a favourable environment for their survival and differentiation. Here, we designed a composite hydrogel composed of collagen 1, an extracellular matrix protein widely used in several therapeutic applications, which we associated with a physical hydrogel generated from a synthetic small amphiphilic molecule. This composite showed improved mechanical and biological characteristics as compared with gels obtained from each separate compound. Incorporation of the physical hydrogel prevented shrinkage of collagen and cell diffusion out of the gel, and yielded a gel with a higher elastic modulus than those of gels obtained with each component alone. The composite hydrogel allowed cell adhesion and proliferation *in vitro*, and long-term cell survival *in vivo*. Moreover, it promoted the differentiation of hASCs in the absence of any osteogenic factors. *In vivo*, cells embedded in the composite gel and injected subcutaneously in immuno-deficient mice produced lamellar osteoid tissue and differentiated into osteoblasts. This study points this new composite hydrogel as a promising scaffold for bone tissue engineering applications.

Keywords

Bone, Tissue engineering, Hydrogel, Osteogenic, Biomaterial, Stem cells, Scaffold

Introduction

Bone injuries and defects are serious health problems, especially those caused by complex fractures or bone defects arising from malformation, osteoporosis and tumors. Severe bone lesions cause hundreds of millions of surgical procedures each year around the world. Lesions which are too large to self-heal require the use of autografts, allografts or grafting of exogenous biomaterials which ensure mechanical stability and provide the appropriate environment for efficient healing[136,137]. However, autographs are often associated with tissue morbidity and present limitations in terms of the obtention of sufficient tissue, and immunogenic rejection and risk of infection are still an unsolved question in the case of allografts[138]. In the case of the use of biomaterials and bone substitutes, limitations have been reported related to poor vascularization leading to poor integration and insufficient bone repair. As such, in some cases, the biomaterial alone is insufficient and exogenous cells must be associated to the biomaterial, as to further support bone regeneration. Moreover, solid biomaterials such as ceramics do not easily fit the size and shape of the defect.

Mesenchymal stromal cells are of considerable interest for many therapeutic applications including repair of damaged tissues, restoration of infarcted heart areas, or anti-cancer therapy[370,371]. These cells exert two types of effects: they can directly take part as building block in tissue regeneration, or they can stimulate a number of host responses which ultimately favor tissue repair or, in the case of cancer, inhibit tumor growth. The latter effect is due to the secretion by these cells of a large number of cytokines and growth factors which can have immuno-modulatory, angiogenic and chemotactic effects[371–375]. In the case where MSCs must be grafted locally, as to exert their therapeutic effect, their efficiency largely dependents on their capacity to persist over a sufficient period within the site of implantation or injection. Additionally, many experimental studies in rodents have shown that MSCs have a short life span after, their engraftment, with commonly less than 10% of the grafted cells still present and alive as early as 10 days post grafting[283,284,376,377].

As such, the design of a biocompatible scaffold that allows MSCs survival, hinders their diffusion and drives their differentiation towards the desired cell type is therefore an important challenge to improve the efficiency, and eventually broaden the field of application of these cells. In this context, injectable hydrogels for application in tissue engineering, in the field of orthopedic sciences, produces many benefits[155]. Therapeutic applications of injectable hydrogels include the filling of large bone defects[136]as they can be formed *in situ* and fit the defect shape and geometry[155].

They should be able to create an environment where exogenous cells can generate functional osteoblasts, when the large lesion hinders the efficient recruitment of endogenous host progenitor cells. It must also favor vascularization of the implant, as to ensure cell survival and allow further recruitment of host cells such as osteoclasts. In recent orthopedic research reports, hydrogels have often been used to maintain the actors of bone regeneration (*i.e*. cells, growth factors, hydroxyapatite particles) together, within the bone lesion[378]. For applications in bone tissue engineering, scaffolds should be fully biocompatible, while eliciting a moderate inflammatory reaction by the host tissue, and thus preventing fibrosis, to avoid damages or encapsulation of the graft[158]. It should also be easy to handle, allowing the incorporation of cells and eventually growth factors, without the need for cross-linking agents or ultraviolet light that could damage cells or proteins[149]. Degradable scaffolds are often desired, aiming to the replacement of the exogenous material by a newly synthetized ECM. Finally, and ideally, scaffold should be injectable to allow minimal and less invasive surgery procedures[155].

As an alternative to polymers, a new generation of physically cross-linked hydrogels has emerged. They are obtained by the self-assembly of building block molecules, without the need for a chemical cross-linker. Gel structure is maintained by weak interactions, which may depend on temperature or ionic strength, and confer to the gel the possibility of a reversible gel-sol transition, that may be useful for drug delivery purposes. We previously described the development of new types of hydrogels, obtained from the self-assembly of small amphiphilic nucleotide-based molecules, glycol-nucleo-lipids[120,379,380], that feature minimal cytotoxicity and that can be implanted or injected into animal tissues[123,381]. In particular, gels based on glyco-nucleo-lipids containing a fluorinated carbon chain (GNF) have been shown to be biocompatible, eliciting a moderate inflammatory reaction and reduced fibrosis[123]. Their half-life was evaluated at *circa* 30 days, both *in vitro* and *in vivo*, and we have also shown that these gels supported the survival of adipose tissue-derived mesenchymal stromal cells (hASCs) aggregates. However, cell adhesion to these gels was limited, obviating an efficient and homogeneous colonization by the seeded cells. Several studies have shown the benefit of designing hybrid hydrogels, in order to combine the properties of each component[137,219]. In this context, the use of collagen gels does have a number of benefits: i) collagen is one of the main components of the ECM of native vascular tissues; ii) it is abundantly available and can be easily purified from living organisms; iii) it is non-immunogenic and biocompatible; iv) collagen gels can be directly and uniformly seeded with cells during the fabrication process; v) the collagen network can be actively

remodeled by smooth muscle cells (SMCs) and fibroblasts (FBs)[382]. Indeed, type I collagen is widely used for tissue engineering and tissue regeneration applications[107,383] as it offers a non-toxic, cell-adhesive and tissue-biocompatible environment. In addition, studies were shown that integrin-mediated adhesion to type I collagen enhances osteogenic differentiation of mesenchymal stem cells[384]. However, collagen shows weak mechanical properties and can be rapidly degraded by endogenous proteases. It is possible to stabilize it with crosslinking agents, or by the use of enzymes[165], but it is difficult to control its stiffness.

The aim of this work was to combine GNF and type I collagen to obtain blend hydrogels using a thermo-gelation process. This composite hydrogel was used as scaffold for cell embedding. After *in vitro* characterization, this composite hydrogel was tested in a subcutaneous implantation model to assess its osteo-inductive properties. This scaffold provided a suitable environment for the cells, conferring them long-term viability and driving their differentiation towards osteoblasts.

4.2. Materials and Methods

4.2.1. Gel preparation

The non-ionic GNF amphiphile was produced by Oxeltis (Montpellier, France). GNF solution was prepared at a concentration of 3% (w/v) by dissolving GNF powder (Oxeltis, France) at 65°C in either phosphate buffered saline (PBS), for physical characterization, or in Dulbecco's Modified Eagle's Medium/F12 (DMEM-F12, Thermofischer Scientific, Invitrogen) for gel-cell complex formation. Gel was formed within 15 minutes after cooling the solution to 37°C.

Type I collagen was extracted from rat-tail tendons and solubilized in acetic acid solution (0.02 N) at a concentration of 4 g/L according to a previously described protocol[115]. A sterile buffer solution was prepared by mixing 5× DMEM (70%, v/v), 1 M HEPES (8%, v/v) and 1 N NaOH (6% v/v) in sterile deionized water (16%, v/v). Collagen hydrogel was prepared by mixing 50% (v/v) sterile collagen solution with 25% buffer solution (v/v) and 25% (v/v) of 1× DMEM at 4° C.

GNF-collagen hybrid hydrogels were prepared by mixing GNF and collagen solutions at 37°C at a 1/1 volume ratio obtaining a final blend solution composed of 0.2% w/v collagen and 1.5% w/v GNF.

4.2.2. FTIR analysis

Interactions of GNF and collagen were investigated by analyzing the FTIR spectra of collagen (2 mg/mL), GNF (1.5% w/v) and the blend (50/50 with collagen at 4 mg/mL and GNF at 3% w/v) solutions. Twenty microliters of the different solutions were deposited on Si crystal. Then, the measurements were performed by using Attenuated Total Reflectance mode (ATR) in FTIR (Agilent Cary 660 FTIR, Agilent technologies, Australia), equipped with a deuterated L-alanine-doped triglycine sulfate (DLa-TGS) detector and a Ge-coated KBr beam splitter. All spectra were corrected for the buffer spectral contribution by standard spectral subtraction procedures. All spectral manipulations were performed using GRAMS (Galactic Industries Corp., Salem, NH, USA). In order to determine the interactions between GNF and collagen, based on band shifts or/and intensity variations, the FTIR spectrum obtained from the physical blend was thus compared to the mathematical one, done by adding the spectra of collagen and GNF solutions, taking also into account the respective solution concentrations.

4.2.3. Rheology

The viscoelastic properties of the GNF, collagen and blend solutions were analysed by dynamic mechanical measurement using a stress-controlled rheometer (MCR 301, Anton Paar, France) with a cone-plate geometry (diameter 40 mm, angle 2°). Gelation was monitored by applying small oscillatory shear strain (1%) allowing measurement of elastic and viscous moduli (G' and G'', respectively) during time, immediately after dissolution of the compounds (collagen and GNF) or after mixing (blend) as described above. Then the dynamic mechanical properties of the gel were measured in a frequency range from 100-to 0.1 rad/s at room temperature after complete gelation.

4.2.4. Cryo-electron microscopy

To determine the pore and fiber size of the gel matrix (for different hydrogel conditions), cryo-scanning electron microscopy (SEM) analysis was performed, with minimal dehydration of the gel matrix. The hydrogel preparations were mounted on a slit holder and plunged into liquid nitrogen slush. Samples were transferred into the Gatan Alto 2500 (Pleasanton, CA, USA) pre-chamber cooled to -90°C and vacuum was applied. After cutting samples with a cold knife, they were sublimated at -48°C for 10 min and then sputter coated for 120 s with gold-palladium. The samples were then transferred to the microscope cryostage (approximate temperature: -130°C) for imaging. The morphology was characterized by SEM (JEOL JSM- 6700) using SEI and LEI detectors operating at 5 kV accelerating voltage, with approximately 8 mm working distance and

the magnifications varied between 250x and 50 000x. The thickness of fibres was measured from SEM images, using Image J.

4.2.5. Cell isolation and culture

Human adipose tissue-derived mesenchymal stromal cells (hASCs) were isolated and cultured as previously described[385] with minor modifications. Briefly, human adipose tissue was obtained from patients undergoing liposuction. Tissue samples were minced and digested in a solution of 0.1% (w/v) collagenase type I (Worthington, Lakewood, NJ, USA) for 1.5h at 37°C with vigorous shaking. After filtration and centrifugation, the top lipid layer was removed and the remaining Stromal Vascular Fraction (SVF) was treated for 10 min with ELB (Erythrocyte Lysis Buffer; 155 mM NH4Cl (Sigma-Aldrich, St. Louis, MO, USA), 5.7 mM K2HPO4, 7.4 mM K2HPO4-3H2O, 0.1 mM EDTA (all Sigma-Aldrich)) and then centrifuged. The pellet was re-suspended in basal medium (BM): DMEM F12 medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Lonza, Basel, Switzerland) and sequentially filtered through 100, 70 and 40 µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Culture medium was replaced every three days. Cell culture was carried out in controlled atmosphere (100% humidity, 37°C, 5% CO_2). The number of passages was less than 6 for all experiments.

4.2.6. Lentiviral transduction

For *in vitro* studies, cells were tagged with either Green Fluorescent protein (GFP) or with Firefly Luciferase (Luc), using lentiviral infection. The GFP or luciferase genes were under the control of the viral MND promoter. For viral transduction, 2.10^5 freshly trypsinised hASCs were mixed with 6.10^6 viral particles (MOI = 20 for MND-Luc and MOI = 30 for MNF-GFP). After 24h in culture, virus-containing medium was replaced by fresh medium and cells were allowed to grow. Medium was changed every day for two days. Cells were then amplified and used for *in vitro* and *in vivo* assays. Expression of GFP was observed under a fluorescent microscope (Zeiss Axiovert 25 CFL microscope; Zeiss, Oberkochen, Germany), with excitation and emission maxima equal to 488 nm and 509 nm, respectively. Luminescence detection was performed after adding of luciferin substrate (Promega) using an EnVision multilabel plate reader (Perkin Elmer, France).

4.2.7. Formation of gel-cell complexes

Three cellularized hydrogel systems were studied: A) GNF gel-cell complex; B) Collagen gel-cell complex; C) GNF/Collagen blend-cell complex. For A, to entrap cells within the gel, 1 mL of the GNF solution was first cooled down to 37°C, then immediately mixed

with 10^6 cells by gentle pipetting. For B, 1 mL collagen hydrogel was prepared on ice as described before but using DMEM containing 10^6 cells. For C, 500µL of collagen-cell complex (solution B) was incubated for 30 minutes at 100% humidity, 37°C and 5% CO_2. 500 µL of cooled (37°C), still liquid GNF hydrogel (3% w/v) was then added and the mixture was gently pipetted four times. The final blend solution was thus composed of 0.2% collagen and 1.5% GNF. Each hydrogel-cell complex (100 µl per sample) was prepared at a final concentration of 10^6 cells / mL of gel.

4.2.8. In vivo *subcutaneous implantation and collection of biopsies*

Bilateral subcutaneous injections were performed on 10-week-old female NOG-SCID mice (central animal facility of the Université Bordeaux Segalen, Bordeaux, France). Luciferase-tagged hASCs mixed with the composite solution were directly injected into dorsal subcutaneous sites using a 21-gauge needle, under gas anaesthesia (isoflurane). Six mice were used for each condition. All procedures and the animal treatment complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. The studies were carried out in accredited animal facilities at the University of Bordeaux, and were approved by the Animal Research Committee of Bordeaux University (Ethical approval number APAFIS#4375-2016030408537165 v4).

4.2.9. Observation and quantitative analysis of gels and cells.

Luc-tagged and GFP-tagged cells were used to monitor the fate of hASCs in a non-invasive manner. Large field observations of gel-cell complexes were performed on Leica fluorescence stereomicroscope (MZ10F, Leica, Germany). These acquisitions were subsequently analyzed to assess hydrogel shrinking using Image J software. For the *in vitro* longitudinal follow-up of cell culture, the GFP fluorescence was measured at 488 nm excitation and 509 nm emission using an EnVisionmultilabel plate reader (Perkin Elmer, France). Fluorescence acquisitions were performed after 6 hours, 2, 7 and 14 days of culture. In living mice, luminescence of cells was measured using the PhotonImager CCDcamera (Biospace®, France). Mice were maintained under anaesthesia by isoflurane inhalation and injected intraperitoneously with luciferin (Promega). Measurements started immediately after substrate injection and the results correspond to the values measured when the signal is optimal and stable for at least 3 min. The signal was integrated for 100 sec and values are expressed as p/s/sr (photons/second/steradian). Measurements were performed during 1 month. All fluorescence and luminescence measurements were compensated for the background noise of the cell-free composite hydrogel (negative control).

4.2.10. Assessment of osteoblast differentiation and of bone matrix production.

Osteoblast differentiation was evaluated by revealing intracellular alkaline phosphatase activity, and extracellular calcium and phosphate within the extracellular matrix. Gel pieces were fixed with 4% paraformaldehyde (PFA) during 20 min at 4°C. Alkaline phosphatase activity was revealed as previously described (Ackerman, 1962). To stain calcium deposits, samples were incubated in alizarin red (Sigma-Aldrich) (2% in 0.1 M acetic acid) for 5 min and washed five times in PBS. To stain phosphate deposition, Von Kossa assay was performed: phosphate containing mineral was revealed by incubating gel pieces in 2.5% silver nitrate for 30 min, followed by two 10 minutes washing steps in water. The samples were observed using an Eclipse 80i microscope (Nikon, Japan).

4.2.11. Histological and immunohistochemical analysis

60 days after gel grafting, the mice were sacrificed using carbon dioxide. Subcutaneous pockets were extracted, fixed in 4% PFA for 4h at 4°C, dehydrated and embedded in paraffin. 4 µm thick sections were de-parafinized using toluene, rehydrated in decreasing concentrations of ethanol (100-50%), washed in distilled water and finally stained with Mayer's hemalun and Erythrosine Masson's trichrome.

To reveal osteoblast differentiation, fixed gel pieces were dried and embedded in Freeze Gel tissue freezing medium (Labonord, France). Frozen sections of 5 µm were cut using cryostat (Leica, Germany), mounted on gelatin coated Star Frost glass slides (Knittelgläser, Braunschweig, Germany) and used for cytochemistry and immunofluorescence. Von Kossa positive mineralization was revealed by incubation of frozen sections in 2.5% silver nitrate for 30 min, then in water for 10 min. Osteocalcin was revealed by immunofluorescence, using anti-human osteocalcin antibody (1/100) (Abcam, UK) as primary antibody, followed by Alexa 568 fluorescent secondary antibody (1/200) (Invitrogen/Life Technologies, USA). The samples were observed using a Eclipse 80i microscope (Nikon, Japan).

4.2.12. Statistical analysis

All statistical analysis were performed using Graphpad Prism 5.0 software. For *in vitro* longitudinal follow-up of GFP fluorescence, measurements were performed on six different wells per condition. For *in vivo* measurement of luminescence, six mice received a Gel-Cells complex. Assessment of statistical significance of differences observed between time points of kinetics was performed using the paired non-parametric Wilcoxon test.

4.3. Results

4.3.1. Physical and structural characterization of the composite hydrogels

As observed in **Fig. 18A1**, FTIR analysis of collagen clearly exhibited the expected spectrum. Indeed, amide bands type I and II from collagen were at 1656 and 1554 cm^{-1}, respectively. Based on the GNF structure, the characteristic bands from fluorocarbon chain were also detected (**Fig. 18A1)**, with a main band from CF_2 symmetric stretching at 1201 cm^{-1}. The band at 1146 cm^{-1} is associated to v_{C-O} from glycosyl, whereas the peaks from 1698 to 1551 cm^{-1} are associated respectively to the carbonyl ($v_{C=O}$) at 1698 cm^{-1} from N-C(=O)-N, to the amide one at around 1650-1635 cm^{-1}, and to C-N and C=C vibrations around 1500-1600 cm^{-1}. The resulting spectrum from the blend exhibits the bands previously detected in GNF and collagen spectra with slight shifts. Indeed, the peak from GNF at 1635 cm^{-1} is shifted to higher wavelength, 1638 cm^{-1}, and the amide I band from collagen from 1656 to 1658 cm^{-1} (**Fig. 18A1**). These shifts could be correlated to chemical interactions such as hydrogen bonding between carbonyl and nitrogen species, as already described in the literature[386]. In order to confirm these observations, the theoretical spectrum (addition of collagen and GNF spectra as described in experimental part) was compared to the experimental one as seen in **Fig. 18A2**. Differences between both are still detected: shifts of the bands but also intensity variations (**Fig. 18A2**). Indeed, the amide II band intensity, 1554 cm^{-1} from collagen, was lower than expected and on the contrary, the intensity of the CF_2 band (1201 cm^{-1}) from GNF, was higher. These discrepancies could be associated to a non-homogeneous blend with clusters richer in GNF than collagen, as previously observed by microscopic analysis. Despite this fact, as slight band shifts are observed, some interactions between GNF and collagen occurred which could induce a change in mechanical properties.

Thus, rheological tests were carried out to compare the visco-elastic properties of gels formed with individual or combined compounds. As shown in **Fig. 18B**, the elastic modulus of type I collagen hydrogels reached a plateau value within 30 minutes, indicating the time necessary to reach a stable mature state. The plateau value was 10 Pa, characteristic of a weak gel (**Fig. 18B)**. GNF-based formula reached a plateau value of 1 000 Pa after 20 minutes (**Fig. 18B)**. The collagen/GNF composite gel had a gelling kinetics similar to the one of GNF, with a plateau value of 2000 Pa (**Fig. 18B)**. The differences of plateau values between the three types of gels were obtained at different frequencies. In summary, the presence of GNF increased the elastic modulus of pure collagen by around 100-fold. Moreover, the combination of collagen and GNF provided

a higher G' value than the one of pure GNF, indicating a synergic effect of this combination to produce a stiffer gel.

We next examined the structure of pure collagen and pure GNF gels by cryo-SEM (**Fig. 18C**). At low magnification (250×), collagen-based gels appeared as a dense, homogeneous fibrous network composed of interconnected fibres forming interconnected pores (**Fig. 18C1**). Higher magnification observation (20000×) showed fibres of different thicknesses, with a mean diameter of 115 ± 42 nm (**Fig. 18C2**). GNF-based gels showed a less dense alveolar structure with bigger interconnected pores (**Fig. 18C3**). At higher magnifications, the walls of the alveoli were showed to consist of a very dense network of overlapping fibres whose thickness was extremely regular (31 nm ± 5 nm) (**Fig. 18C4**). Walls were connected by thick trabeculae resulting from the convergence and aggregation of fibres that often displayed a twisted aspect. In the blend collagen-GNF composite gel, contacts between the GNF walls and extensions from the collagen network were observed (**Fig. 18C5**). Such a pattern supports the notion that blending resulting in a physical mixture of both components, without the formation of a third different phase deriving from interactions between collagen and GNF fibers (**Fig. 18C6**).

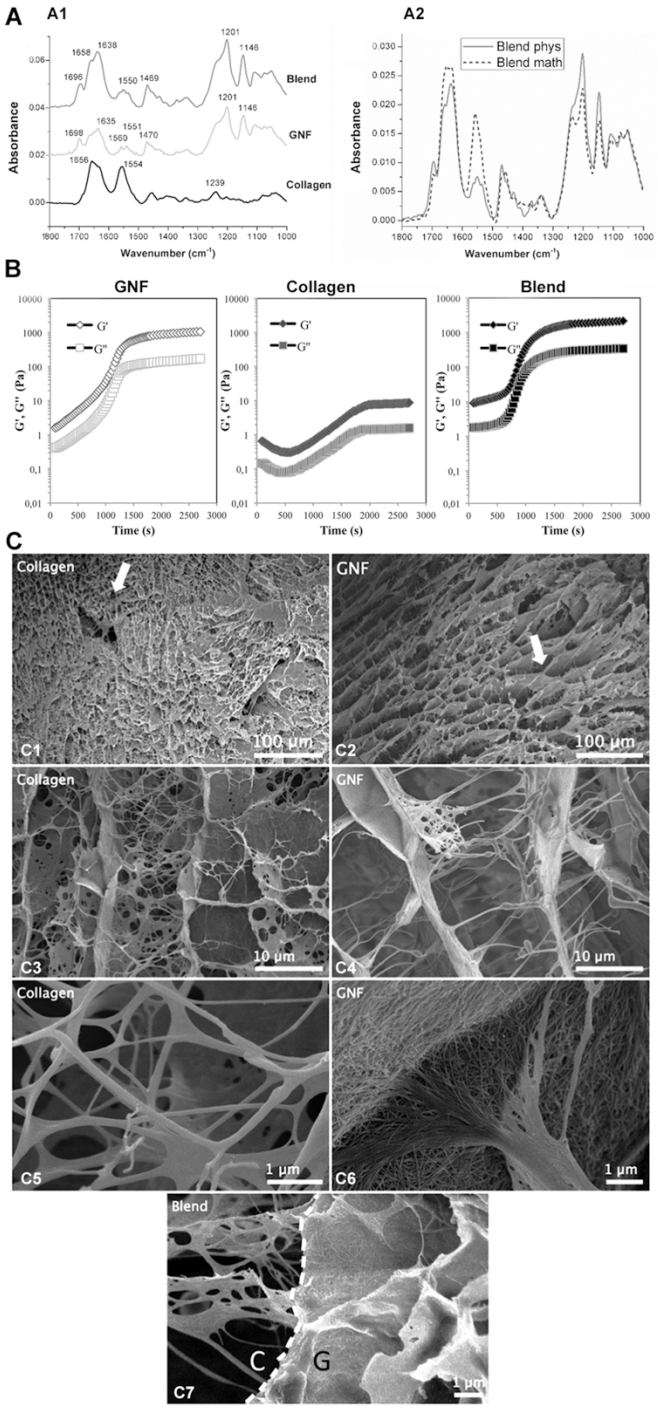

Figure 18 : Material, rheological, structural characterization

(A1) FTIR spectra of collagen hydrogel (black line), GNF hydrogel (light grey line) and collagen-GNF blend hydrogel (heavy grey line) in the 1000-1800 cm-1range. (A2) Comparison of the theoretical curve obtained from the mathematical combination of GNF and collagen curves (dotted grey line), with the real curve obtained with the blend (plain grey line). (B) Characterization of the rheological properties of GNF, collagen and GNF-Collagen blend hydrogels. Time course of elastic (G') and viscous (G'') moduli of GNF, collagen or blend systems were followed immediately after preparation. (C) Microstructure of hydrogels was assessed by cryo-electron microscopy. Pores are shown on low magnification pictures (white arrows). The separation between GNF and collagen gels in the blend composite hydrogel is shown as a white dashed line. Scale bars are shown at the bottom of each image. G and C stand for GNF and collagen moieties, respectively.

4.3.2. In vitro *interactions between hydrogels and hASCs*

hASCs were grown for 7 days in either collagen, GNF or collagen-GNF blends. As shown in **Fig. 19A**, when hASCs were seeded in collagen hydrogels, a strong shrinkage of the cellularized material was observed already 48 hours after seeding. In contrast, the sizes of cellularized GNF and of collagen-GNF blend hydrogel remained constant. This difference in contraction was even higher at 7 days (**Fig. 19B**): no significant shrinkage could be observed when collagen was blended with GNF, while plain cellularized collagen gels showed a surface reduction of approximately 70%. Whereas many cells spread out of the shrinking collagen gel and adhered to the cell culture dish (**Fig. 19C**), no such leak was found in the presence of GNF in blend configuration (data not shown). Given its poor mechanical properties and its failure to retain embedded cells, we did not select pure collagen as a scaffold for further *in vitro* and *in vivo* studies.

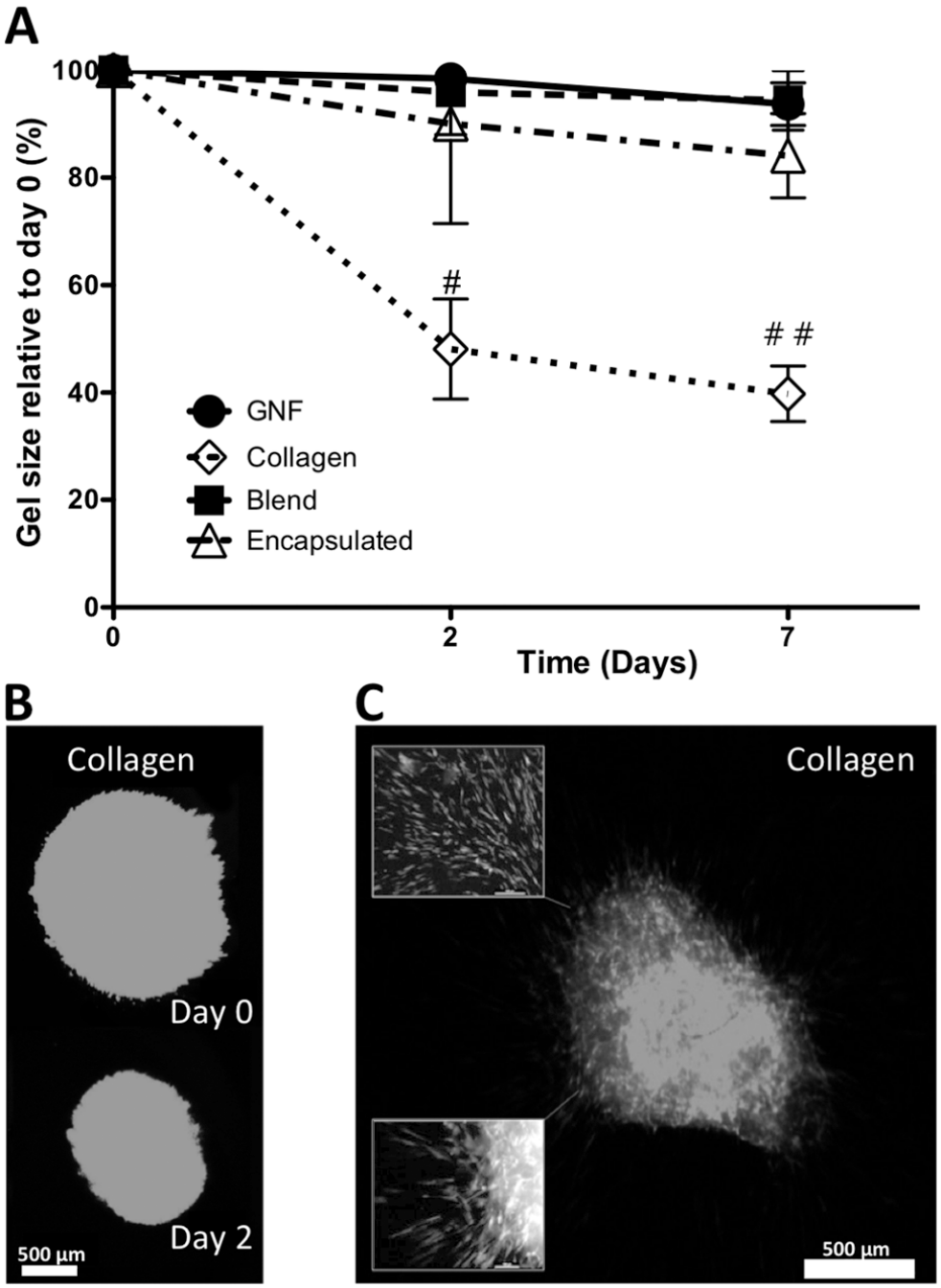

Figure 19 : Hydrogel compaction

(A) Evaluation of collagen hydrogel compaction when hASCs were cultured in collagen alone (diamonds, dotted line), GNF alone (circles, plain line), collagen blended with GNF (squares, dashed line). % of initial area was measured at different times after cell seeding (Average+/-SD, n=6); # denotes significant differences between collagen and Blend hydrogel and GNF hydrogel ($p<0.05$); # # denotes significant differences between collagen and Blend hydrogel and GNF hydrogel ($p<0.001$). (B) Pictures of collagen scaffolds containing hASCs 1 hour and 2 days after seeding; scale = 500 µm(C) Examination by confocal microscopy of collagen gels cellularized with hASCs at day 7. Inserts: cells escaping the collagen matrix and adhering to the surface of the plastic cell culture dish.

4.3.3. In vitro *adhesion and proliferation of hASCs*

hASCs encapsulated either in pure GNF or blend hydrogels are presented in **Fig. 20**. When hASCs were contained into blend gels, cells attached within the gel after less than 6h (**Fig. 20A**, D0) as well as hASCs entrapped into 1.5% GNF scaffolds. After 2 days, adhesion and spreading of hASCs were observed in blend hydrogels and were similar to cells seeded on polystyrene dishes (adhesion positive control). In GNF hydrogels the signal decreased sharply between the second and the fourteenth day (**Fig. 20B**) and the observation of cell morphology by microscopy showed a few round-shaped cells (**Fig. 20A**). In GNF-collagen blend hydrogels, the significant difference in the intensity of the signal between days 2 and 14 reflected cell proliferation within these hydrogels. Finally it was observed that the addition of collagen to GNF significantly improves the biological properties of the hydrogel as cell culture support.

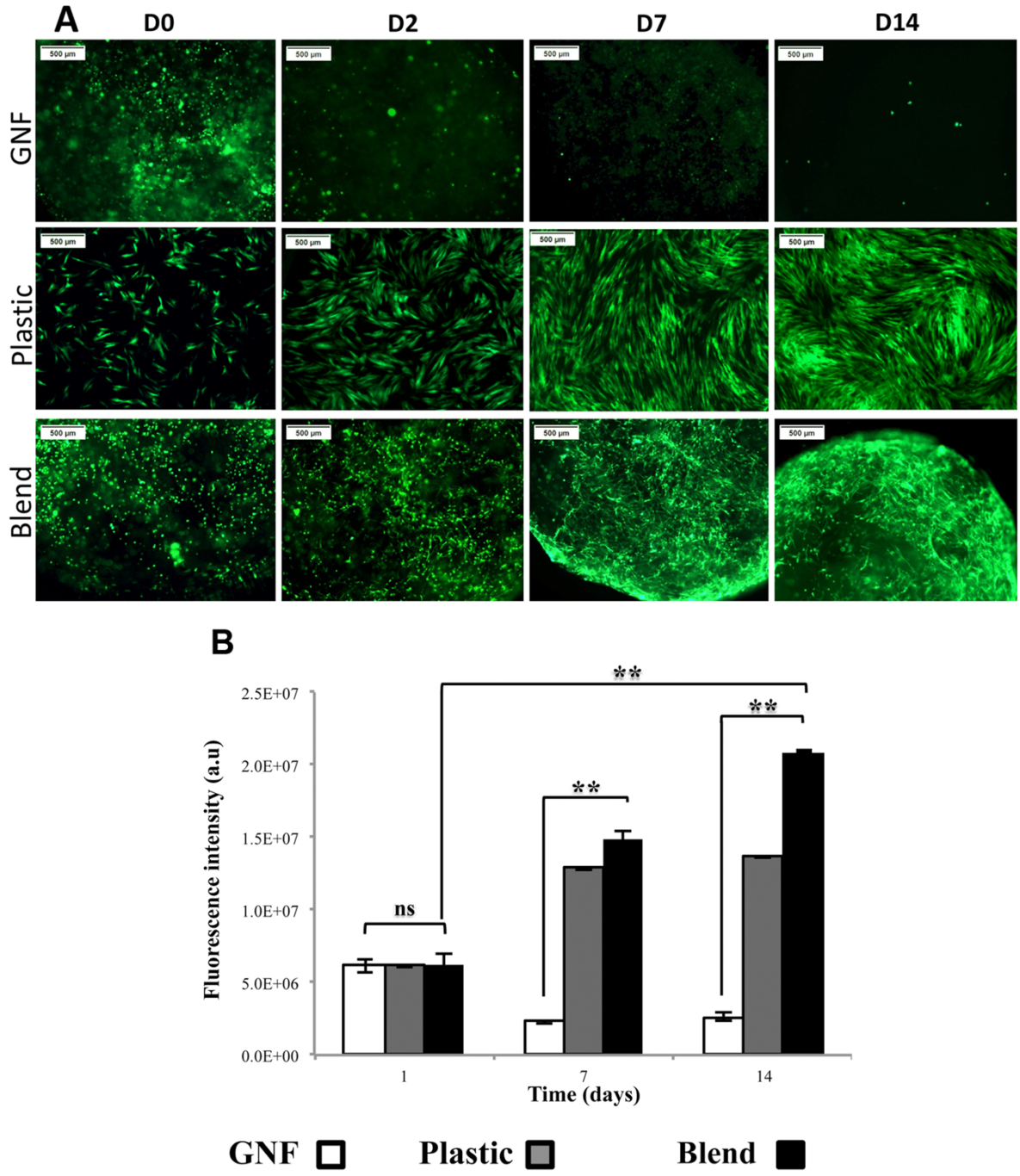

Figure 20 : Cell proliferation

*3D cell culture using GNF-based and composite hydrogels as scaffolds. GFP-tagged hASCs were observed (A, Scale bar = 500 µm) and quantified by measuring GFP fluorescence (B, arbitrary units) after 6 hours, 2, 7 and 14 days of seeding. Cells were either encapsulated either in GNF-hydrogel (white bars) or seeded onto polystyrene culture dish (grey bars) or in GNF/collagen blend hydrogel (black bars). In B, results are expressed as mean +/- standard deviation. Statistical significance of difference was assessed by Mann-Whitney U-test; *: $p<0.05$; **: $p<0.01$.*

4.3.4. In vivo fate of cellularized constructs

To challenge the benefits of GNF-collagen blend hydrogel compared to GNF hydrogel for cell grafting efficiency, the two kinds of gels were seeded with hASCs expressing the firefly luciferase gene and implanted into the dorsal subcutaneous region of NOG-SCID mice. Cell growth was monitored non-invasively by measuring Luc luminescence emitted from the area of implantation (**Fig. 21A**). When cells were injected within the GNF gel, the fluorescence decreased rapidly over time (**Fig. 21B**). Thirty days after implantation, only 13% of the initial signal was still persistent in GNF hydrogels while 213% of the initial signal was detected in the blend condition.

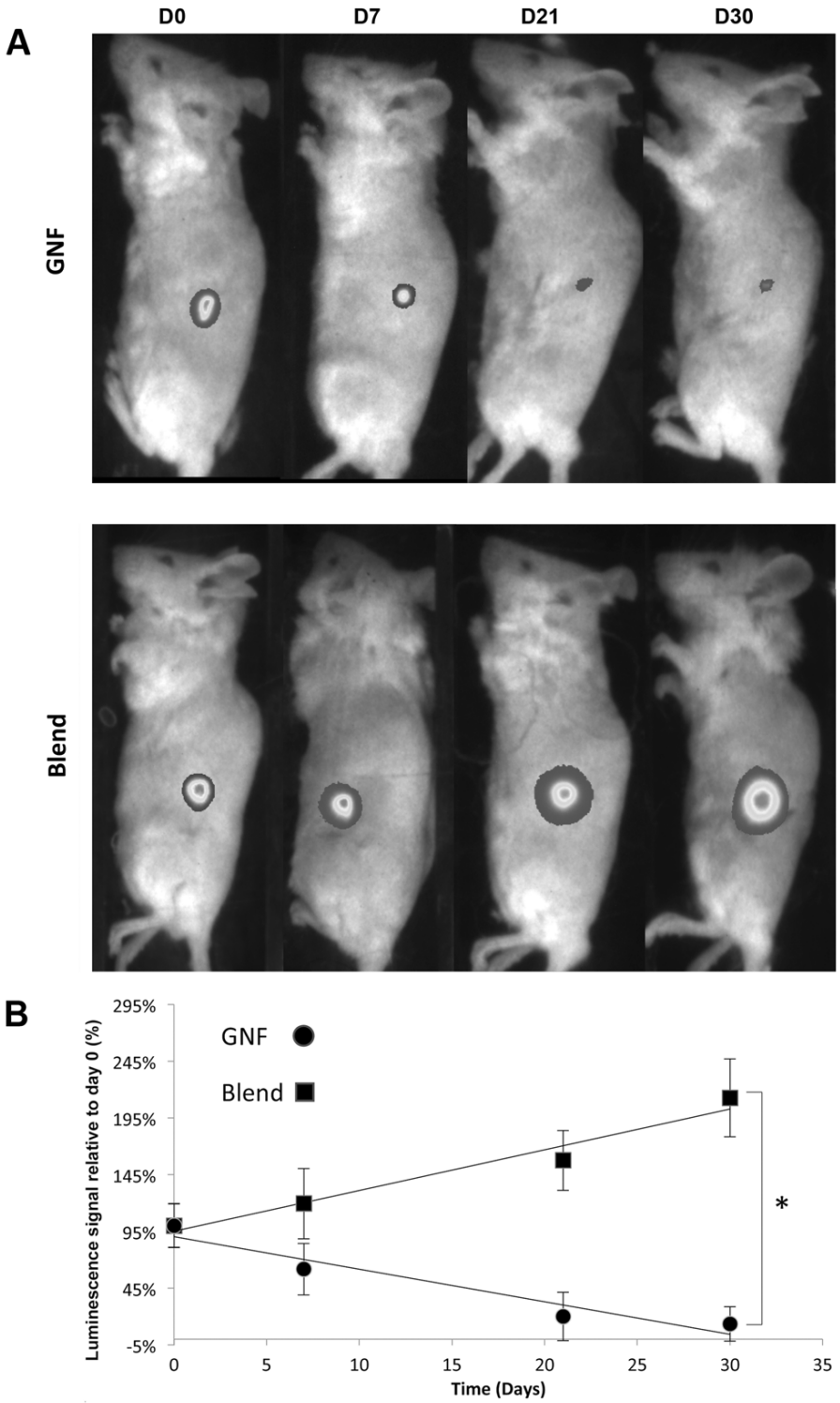

Figure 21 :In vivo cell imaging

*In vivo imaging of Luc-tagged hASCs included in GNF-based or hybrid gels and subcutaneously injected in NOG-SCID mice. Luminescence from either GNF-hASCs complex (circles) or blend-hASCs complex (squares) is observed (A) and quantified during 1 month (B). Statistical significance of difference was assessed by Mann-Whitney U-test *: $p<0.05$.*

4.3.5. In vitro and in vivo differentiation and fate of hASCs-hydrogels complex

After showing that the GNF-collagen blend hydrogel enables hASCs survival *in vitro* and *in vivo* and maintains them in the implantation site, we examined whether it could influence cell differentiation. Fourteen days after the onset of the culture in basal medium (BM), a significant proportion of alkaline phosphatase (ALP) positive cells were observed among hASCs dispersed inside blend hydrogels, whereas very few positive cells were found in the GNF gel (**Fig. 22A**). Extracellular matrix was weakly positive for alizarin red staining (**Fig. 22B**) and Von Kossa (**Fig. 22C**) only in blend hydrogels, revealing partial mineralization of the matrix and suggesting the deposition of calcium phosphate.

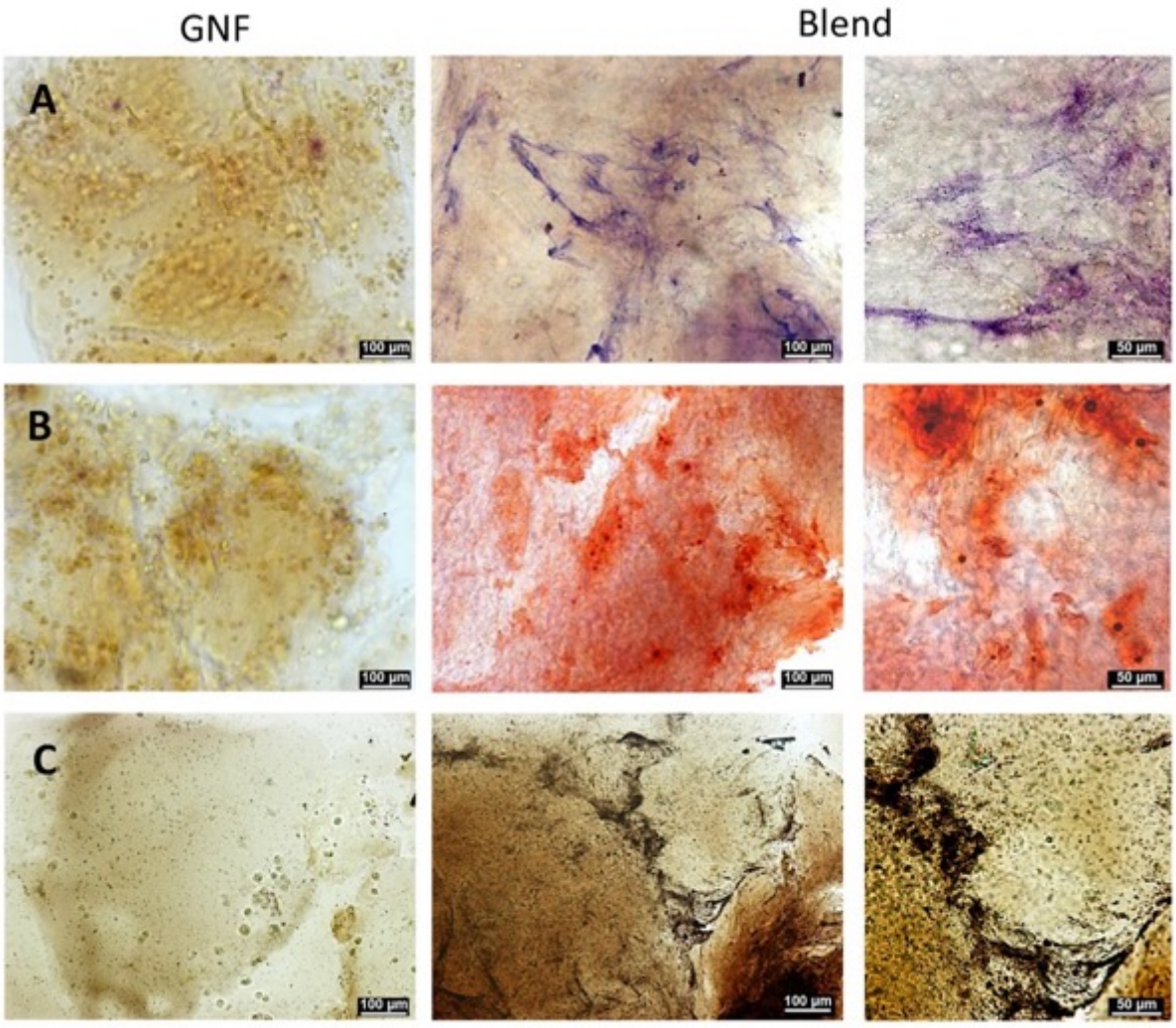

Figure 22 : In vitro production of mineralized extracellular matrix by hASCs encapsulated in hybrid hydrogels.

hASCs encapsulated either in GNF-based hydrogel (GNF) or in GNF-collagen 1 hydrogel (blend) are cultured in basal medium during 14 days. (A) Expression of alkaline phosphatase is assessed by purple staining. Synthesis of a mineralized extracellular matrix is revealed by Alizarin Red (B) and Von Kossa staining (C) (x10 magnification, scale bar = 100 µm ; x20 magnification, scale bar = 50 µm).

When cell-free GNF-collagen blends were implanted in mice, after 60 days, Masson's trichrome staining showed the persistence of a few hydrogel blocks, surrounded by fibrous tissue, but did not reveal the presence of any osteoid tissue (**Fig. 23A**). In contrast, tissues in which cellularized GNF-collagen blend hydrogels had been implanted showed, in addition to few gel blocks, several circular bone-like structures, showing lamellar organization of the collagen, and containing osteocyte-like cells (**Fig. 23B**). These structures were strongly reactive to Von Kossa staining, confirming their high degree of mineralization (**Fig. 23C**). The presence of osteoblasts/osteocytes within the mineralized areas was confirmed by the strong labeling obtained with anti-osteocalcin antibodies (**Fig. 23D**). Moreover, since the antibodies were directed against human osteocalcin, these data support the persistence of gel-embedded human cells even 60 days after their engraftment.

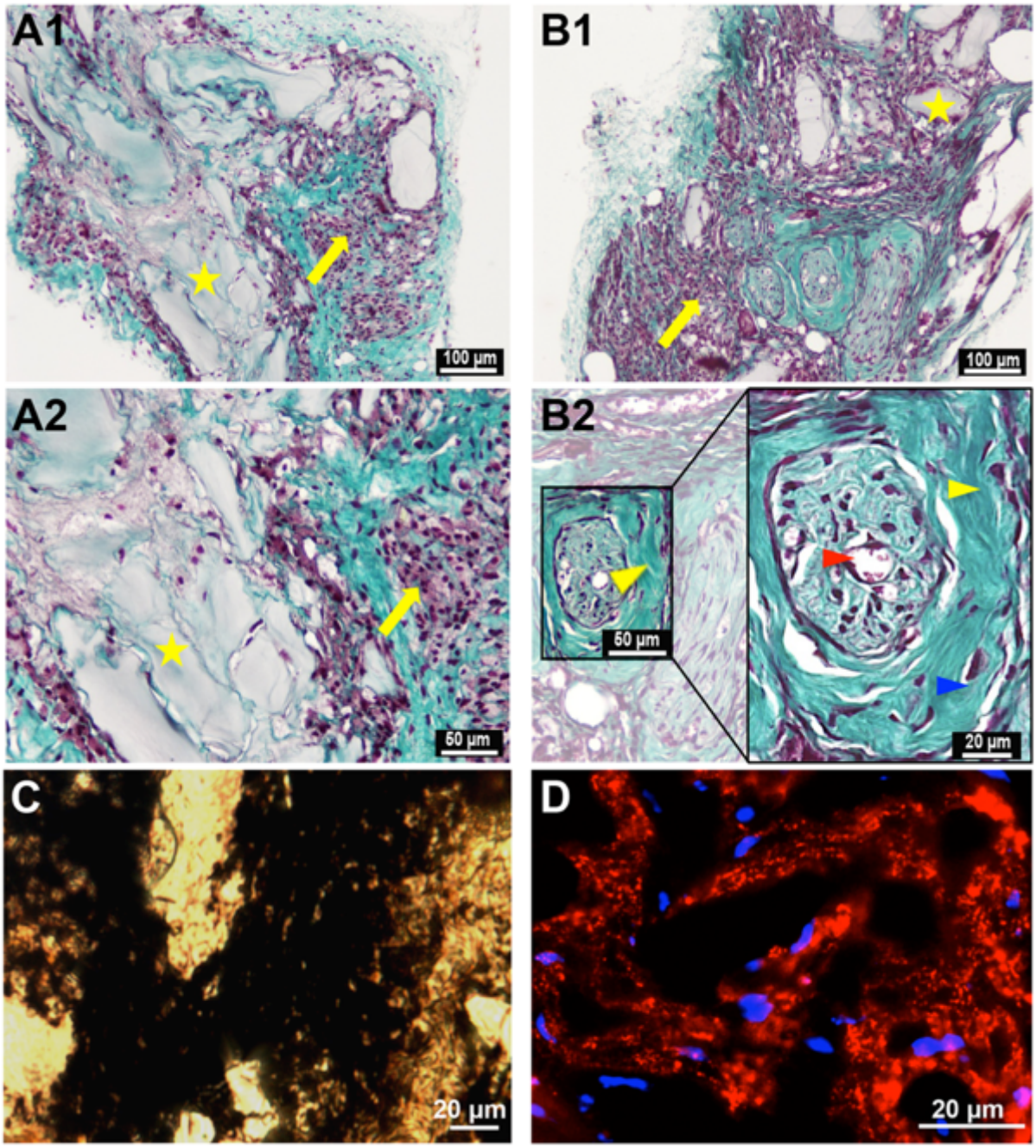

Figure 23 : In vivo analysis

Histological Analysis of biopsies 60 days after subcutaneously injection in NOG-mice of collagen-GNF blend hydrogels without (A) or with (B) hASCs. Paraffin sections of biopsies were stained with Masson's trichrome and observed at x10 (A1, B1), x20 (A2, B2) and x40 (B2 insert) magnification (Scale bar = 100 µm, 50 µm, 20 µm respectively). Stars: gel blocks; Arrows: fibroblastic cells invading the hydrogel; Yellow arrowheads: organized extracellular matrix (ECM); Red arrowhead: blood vessel containing hematies; Blue arrowhead: cells entrapped in ECM (B2). Phosphate-rich ECM is revealed by Von Kossa staining (C). Differentiation of hASCs is shown by red positive osteocalcin staining, nuclei are stained in blue by DAPI (D) (Scale bars = 20 µm).

Discussion

This study provides support for the potential therapeutic interest of a composite hydrogel based on the combination of type I collagen with a recently described temperature-dependent gel-forming synthetic compound. Data include improved mechanical stability and stiffness, *in vitro* adhesion and expansion of embedded mesenchymal stromal cells, and *in vivo* persistence of grafted cells. In addition, these data reveal the osteogenic property of this composite.

The use of a scaffold is imperative when considering the admnistration of exogenous cells for therapeutic applications like bone tissue engineering, as it can support and even orchestrate their survival and function, once implanted in host tissues. As such, the scaffold must ensure cell survival and retention at the implantation site, until grafted cells have exerted their paracrine effect, or until a new ECM is synthetized. At this step the biomaterial becomes dispensable and should be degraded to make room for new tissue.

Collagen is a well established natural biomaterial. As one of the major components of the ECM, biodegradable and biocompatible, it is an obvious choice for the development of matrices for tissue engineering applications[107,382]. Nonetheless, it presents weak mechanical properties and has shown fast degradation rates, both *in vitro* and *in vivo*. Our data confirmed these limitations: 1) rheological characterization of collagen gels yielded a 10Pa elastic modulus, in line with previous studies[387]; 2) the contraction of the cell-seeded collagen matrix, which may result in cell damage, reduction of nutrient diffusion and cell loss is again in agreement with other authors[388].

As means to circumvent these limitations, and while maintaining the favorable cell-adherence properties of collagen, we combined collagen with glycosyl-nucleosyl-fluorinated (GNF) molecules. GNF-based gels show an elastic modulus of 1000 Pa, in agreement with previous studies[123]. When blended with collagen, the elastic modulus of the composite gels exceeded the value observed for GNF alone, establishing the structuring role of this synthetic component. The mechanical role of GNF was further assessed by its capacity to prevent collagen shrinkage, when both compounds are associated. This stabilizing effect may result from interactions formed at the interface between collagen and GNF, as suggested by cryo-scanning electron microscopy images. Such interactions may counteract the retraction forces exerted by the embedded cells and equally restrict the diffusion of cells outside the implanted material. Additionally, preservation of cells within the injection site may also result from the protection against tissue proteases, delaying collagen degradation and enabling cells to produce and deposit their own matrix. We can conclude from these data that GNF plays a crucial role

in the structuration and stabilization of the composite gels. Our findings showing the mechanical stabilization of collagen by association with a structuring gel are in agreement with other studies in which collagen was associated with different natural[389] or synthetic polymers[390].

The chemical, physical and mechanical properties, as well as the stability of hydrogels, have an impact on the *in vitro* behavior of hydrogel-embedded cells[391]. Here, we show that human hASCs embedded in pure GNF gels poorly survive as isolated cells, and keep a round morphology that reflects their inability to establish attachment points to the matrix. These observations are in agreement with our previous studies pointing GNF as forming a non-cytotoxic, biocompatible, slowly degrading hydrogel, in which only cell aggregates can survive[123]. When embedded in the composite hydrogel, hASCs survived and proliferated not only *in vitro* but also *in vivo*. *In vitro*, hASCs retained a fibroblastoid morphology of matrix-adherent cells. *In vivo*, cells were found embedded in a collagenic extracellular matrix. Thus, the GNF-collagen blend provides a microenvironment that is favorable to the long-term survival of hASCs, 60 days on our observations. This is a major and original property of this new composite gel. Indeed, several *in vivo* studies, addressing the fate of biomaterial-associated cells after their implantation, have revealed the loss of the majority of cells within 10-14 days[284]. We assume that this behavior is due to the composite gel capacity to by one side provide adhesion sites to cells, within the collagen matrix, while preventing cell migration, due to the GNF fraction. Additionally, the composite structure allows easy diffusion of oxygen and nutrients, does not cause fibrosis and is rapidly perfused by blood vessels that it provides a favorable environment for cells. In summary collagen improved cell survival, whereas GNF conferred mechanical stability, retention of cells within the gel, maintenance of gel shape and cell morphology.

This study reveals another major property of GNF-collagen composite hydrogels: their capacity to promote hASCs differentiation towards the osteobastic phenotype, and to elicit the formation of bone-like structures in an ectopic site *in vivo*. The expression of alkaline phosphatase in a majority of composite-embedded cells and the deposition of a mineralized matrix within this gel clearly demonstrates the massive presence of osteoblasts upon *in vitro* culture. Our previous studies had shown that GNF promoted the osteoblastic differentiation of hASCs spheroids[123]. However, collagen itself has been shown to promote osteoblastic differentiation of MSCs[392], and the present study cannot discriminate between the contributions of both parts. Nevertheless, this new composite hydrogels appears as a promising scaffold for the use of human MSCs for

bone regeneration, as it displays osteogenic properties. Previous studies have shown the benefit of hybrid gels, containing a collagen fraction, for the *in vivo* survival and osteoblast differentiation of MSCs[393]. The great advantage of GNF-based gel over commonly used polymers is its synthetic origin, allowing perfect control of purity and reproducibility, its injectability, which allows minimally invasive grafting procedures and the easy handling of the product, which enables the thorough incorporation of cells and bioactive molecules before injection. Indeed, gel formation is achieved by cooling the hot GNF solution, avoiding any chemical cross-linking that would require potentially harmful reagents. Moreover, jellification occurs slowly, allowing the safe mixing of cells at 37°C before gel formation is completed. This property also offers the possibility to inject the GNF solution and to let it gel *in situ*. Considering all properties of GNF together, this compound appears as a promising structuring and protective biomaterial, which may be combined with other scaffolds. Applications in tissue engineering, for bone defects healing but also for the treatment of other tissue lesions, by associating GNF with a cell-friendly matrix, can be envisaged and will require further studies in orthotropic sites and with the choice of other natural matrices depending on the target tissue.

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5. Prolonged delivery of BMP-2 by a non-polymer hydrogel for bone defect regeneration

Mathieu Maisani[1,2], Sindhu KR[1], Mathilde Fenelon[1,3], Robin Siadous[1], Sylvie Rey[1], Diego Mantovani[2], Olivier Chassande[1]

[1]*Laboratoire BIOTIS, Inserm U1026, Université de Bordeaux, Bordeaux, France*

[2]*Lab. for Biomaterials & Bioengineering CRC-I, Dept. Min-Met-Materials Engineering & Research Center CHU de Québec, Laval University, Québec City, Canada*

[3]*CHU Bordeaux, Services d'Odontologie et de Santé Buccale, Bordeaux, France*

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5.1. Résumé

La protéine morphogénique osseuse 2 (BMP-2) est un puissant inducteur de formation osseuse. La BMP-2 est actuellement utilisé en clinique pour traiter certaines lésions osseuses. L'extension du champ d'application de cette molécule nécessite la conception de systèmes de distribution qui protègent la protéine d'une dégradation précoce et permettent sa libération progressive. Cette étude décrit l'utilisation d'un hydrogel non polymérique, basé sur l'auto-assemblage de glycosyl-nucléolipides amphiphiles comme système de distribution de BMP-2. La BMP-2 peut être facilement encapsulée dans des gels à base de glycosyl-nucléosyl-fluorés (GNF) et lentement libérée *in vitro*, tout en maintenant son activité ostéogénique. Lorsque des hydrogels contenant de la BMP-2 marquée par un fluorophore ont été déposées sur un défaut de calvaria chez la souris, le signal détecté chez les souris vivantes diminue lentement pour être encore détecté après 3 semaines. La protéine encapsulée au sein du gel a favorisé une formation osseuse importante 8 semaines après la chirurgie. L'os nouvellement formé couvrait la plupart des défauts et présentait des caractéristiques histologiques semblables à celles de l'os natif. En revanche, lorsqu'une solution liquide de BMP-2 a été injectée dans les défauts, le signal de fluorescence a diminué rapidement et aucune formation osseuse significative n'a été observée. La propriété unique de l'hydrogel à base de GNF en tant que système d'administration injectable et de matériel ostéogénique a été révélée dans un modèle sous-cutané avec la formation d'os ectopique. Les blocs d'hydrogel GNF chargés de BMP2 injectés ont provoqué la formation d'os spongieux. Celui-ci comportait toutes les caractéristiques typiques de l'os en remodelage et contenait de la moelle osseuse. Ces résultats montrent que cet hydrogel à base de GNF est un système de livraison de BMP-2 efficace et un matériau ostéogène facile à utiliser pour supporter la régénération osseuse.

Abstract

Bone morphogenetic protein 2 (BMP-2) is a potent inducer of bone formation that is currently used in a limited number of clinical indications to treat extensive bone loss. Extending the field of applications of this molecule requires the design of delivery systems that protect the protein from early degradation and allow a slow long-term release. This study describes the use of a non-polymer hydrogel, based on the self-assembly of small amphiphilic glycosyl-nucleolipids into micellar structures, as a new type of delivery system for BMP-2. BMP-2 could be readily encapsulated in glycosyl-nucleosyl-fluorinated (GNF)-based gels and slowly released *in vitro*, while maintaining its osteogenic activity. When hydrogel pieces containing fluorophore-tagged BMP-2 were deposited onto a calvaria defect in mouse, the signal detected in living mice slowly

decreased and was still detected after 3 weeks, suggesting a slow gradual release. Gel-embedded protein promoted significant bone formation 8 weeks after surgery. The newly formed bone covered most of the defect and showed histological features similar to those of native bone. In contrast, when a solution of BMP-2 was injected into the defects, the fluorescence signal decreased rapidly and no significant bone formation was observed. The unique property of the GNF-based hydrogel as an injectable delivery system and osteogenic material was revealed in a subcutaneous model of ectopic bone formation. Injected BMP2-laden GNF hydrogel blocks elicited the formation of cancellous bone, showing all the typical features of remodeling bone that contains bone marrow. These results show that this GNF-based hydrogel is an easy-to-use, efficient delivery system for BMP-2 and osteogenic material to support bone regeneration.

5.2. Introduction

Some bone tissue lesions can result in severe bone loss. If this occurs and the surrounding tissue is also damaged, the damaged bone is inadequately reconstructed so a graft of exogenous tissue is needed to fill the large bone defect. Such grafts function as a mechanical substitute for the lost bone and a template for bone regeneration. The addition of osteogenic factors may promote the recruitment and activation of endogenous progenitor cells to regenerate bone. Since bone grafts involve the limitations of tissue availability and morbidity issues, efforts are increasing to develop alternatives to autografts and allografts by using various biomaterials[394]. These biomaterials have different chemical and mechanical properties depending upon the site of injury, the type of bone needing repair, and especially on the mechanical load that the damaged bone must support. Whereas metallic implants are the first line for hip fractures, ceramics, glasses and pastes consisting of calcium phosphate are widely used for the treatment of many bone lesions such as non-union fractures and in maxillofacial surgery[331]. However, these materials are degraded very slowly and have weak osteoinductivity. Furthermore, they do not lead to the regeneration of new fully functional bone in the case of extensive lesions. An alternative is to use hydrogels to carry osteogenic and/or angiogenic factors in order to promote bone reconstruction[395,396]. Hydrogels are potentially injectable so they allow the tight fitting of the repair material to the size and shape of the defect, thereby ensuring a total interaction with the surrounding tissue[158]. They have the capacity to entrap many different factors such as calcium phosphate particles, which are osteoinductive, stem cells, which may provide the missing osteoblasts and endothelial cells required to regenerate vascularized bone, and growth factors, which can stimulate either gel-embedded or endogenous host cells[136]. The

option of providing growth factor to the damaged bone without the input of exogenous cells constitutes a cheaper, easier and safer therapeutic solution[73].

Bone morphogenetic proteins (BMPs), which belong to the TGFß superfamily, play an important role in the development of many tissues[397], but also in the formation and dissemination of tumors [398]. Among the BMPs, BMP-2 and BMP-7 have been shown to be the most potent osteoinductors. They are naturally involved in bone formation and repair [399] but are also able to induce bone formation ectopically[400]. As they can be easily produced as a stable recombinant protein in bacteria, BMPs have been proposed for the treatment of several pathologies involving severe bone loss, such as vertebral fractures, non-unions and osteoporotic fractures. Although they have been approved by the FDA for clinical use in the orthopedic and dental fields[126,401], their use is still hampered by several drawbacks and undesirable side-effects. To achieve clinically significant bone regeneration, supra-physiological amounts of BMP-2 are needed, which may produce structurally abnormal bone and result in tissue inflammatory reactions[402] and heterotopic ossification[403].

To circumvent these limitations, several systems have been designed to reduce the doses necessary to achieve the therapeutic effect. These strategies aim at controlling the release of BMP over time, limiting the initial burst and prolonging the therapeutic action of the molecule[331]. Most of the systems which have been designed rely on the use of polymers in which chemical modifications have been introduced in the backbone[155]. Release control can be achieved either through weak interactions between the protein and the polymer, resulting in transient retention, or through stimulus-triggered release (pH, temperature, enzyme activity…)[404]. Alternatively, nanoparticles containing BMP-2 have been incorporated in hydrogels as a reservoir and delivery system[336,339,405–407].

All these products involve complex multi-step chemistry involving several reagents. The degradation of these gels is likely to release multiple molecules whose long-term physiological effects are difficult to predict. Recently, new types of hydrogels generated by the self-assembly of amphiphilic molecules have been developed[408]. Some of these gels have been obtained by the use of small, nucleotide-based building blocks[409]. Gels formed by the self-assembly of glycol-nucleolipids[379] have shown promising biological properties such as the absence of cytotoxicity. Within this family, molecules containing a fluorinated hydrocarbon chain have shown a moderate inflammatory response and relatively slow degradation rate that is compatible with the formation of new bone, thereby opening the way for new clinical applications[121,123].

The purpose of this study was to develop a BMP-2 delivery system by embedding BMP-2 in a hydrogel composed of glycosyl-nucleosyl-fluorinated (GNF) amphiphile. The capacity of this gel to entrap human recombinant BMP-2 was assessed and the *in vitro* release kinetics of the protein was established. The promising release profile observed prompted us to challenge this delivery system *in vivo* to promote new bone formation in both a subcutaneous ectopic model and an orthotopic model, using a low dose of BMP-2 embedded in GNF. The *in vivo* release of BMP-2 was monitored by blending the native BMP-2 with a fluorescent dye-tagged molecule. Finally, the capacity of the gel-embedded BMP-2 to enhance bone repair and to promote *de novo* bone formation were assessed in a mouse calvaria defect model and in a subcutaneous injection model, respectively.

5.3. Material and methods

5.3.1. Recombinant human Bone Morphogenic Protein-2 (BMP-2)

BMP-2 (E. Coli-derived) was obtained from PeproTech (USA). Part of the BMP-2 was labeled with a fluorescent tag using a Xenolight 685 kit (PerkinElmer, USA) according to the manufacturer's protocol.

5.3.2. Hydrogel preparation

The non-ionic GNF amphiphile was produced by Oxeltis (France). The GNF solution was prepared at a concentration of 1.5% (w/v) by dissolving GNF powder in a 0.1 M phosphate buffered saline solution (PBS) at 65°C. The GNF solution was incubated at 37°C for 30 min to form a gel. This composition was referred to as "GNF". For the gel-embedded BMP-2, a mixture of native BMP-2 (10µg/ml, dissolved in PBS 0.1 M) and tagged BMP-2(0.5µg/ml) was added to the GNF solution at 37°C before gel formation. This condition was referred to as "GNF-BMP-2". The same BMP-2 mixture when solubilized in 0.1M PBS was named "Liquid BMP-2".

5.3.3. In-vitro data analysis

5.3.3.1. In vitro release

To study the release kinetics, 100 µl of liquid "GNF-BMP-2" were poured into inserts and placed in the 24-well plate containing 500µl of PBS after gel formation. The well plates were incubated at 37°C for 14 days in a shaking incubator at 100 RPM. At predetermined times (0h, 3h, 6h, 9h, 24h, 48h, 72h, 168h (1 week) and 336h (2 weeks)), the supernatant was collected and replaced with fresh PBS (0.1 M). Fluorescence was measured using fluorescence imaging (Lumina LT device, Perkin Elmer, Massachusetts, USA) at 675nm (excitation) and 694nm (emission) wavelengths to quantify the amount of fluorescent

BMP-2 released in the supernatant. The supernatant was then stored at -80 °C until being processed for ELISA assay. At the end of the experiment, all collected supernatants were thawed. The BMP-2 concentration in each sample was assessed using a Human BMP-2 Standard ABTS ELISA Kit (Peprotech, USA) according to the manufacturer's protocol. This measurement was performed 6 times at each time point. The cumulative amount of released BMP-2 was calculated and plotted as a function of time. Results were expressed as an average ± standard deviation.

5.3.3.2. In vitro bioactivity

To determine the biological effect of released BMP-2 on osteogenic differentiation, human adipose-derived stem cells (hASCs) obtained from liposuction surgery (ethical approval # DC-2008-412) were seeded on 24-well culture dishes ($2x10^4$ cells/well). Fifty µl of "GNF-BMP-2" were placed onto the top of transwell membranes. Culture medium (DMEM F12, Life Technologies, USA) was added to each well. In parallel, other cells were incubated in osteogenic medium (DMEM F12 + StemPro® osteogenesis differentiation kit, Life Technologies, USA) as a positive control. For qualitative assessment of alkaline phosphatase activity, hASCs were fixed after 7 days of culture with 4% paraformaldehyde for 30 minutes. Wells were washed twice with PBS and processed using the Alkaline Phosphatase kit 86 C-1KT (Sigma-Aldrich, USA), as described in the manufacturer's instructions. Briefly, cell layers were incubated with Fast Blue RR salt and Naphtol AS-MX Phosphate Alkaline solution 0.25 % (Sigma-Aldrich, USA) for 30 min at room temperature in the dark. Samples were analyzed using a stereo microscope (MZ10F, Leica Microsystems, Germany) coupled to a camera (Leica model DFC 450C). For quantitative analysis of ALP, proteins were extracted by cell lysis in distilled water at 4 °C. Protein content was quantified according to Lowry's method[410]. In parallel, ALP activity was determined after incubation of protein extracts in the presence of para nitrophenyl-phosphate for 30 minutes at room temperature according to the kit manufacturer's recommendations (Wako Pure Chemical Industries, VA, USA). The absorbance of the produced p-Nitrophenol was measured at 405 nm using a Victor ® spectrophotometer. ALP activity results were expressed as nmol of p-Nitrophenol/ µg of protein as an average ± standard deviation. This analysis was performed 6 times.

5.3.4. In vivo animal models

The study protocol was performed according to the Institute of Animal Care guidelines formulated by the National Society for Medical Research. The studies were carried out in accredited animal facilities at the University of Bordeaux (accreditation number: A33-063-917) and were approved by the Animal Research Committee of Bordeaux University (Ethical approval number APAFIS#4375-2016030408537165 v4). Eight-week-old OF-1

female mice (Charles Rivers, France) were used for this study. Mice were anesthetized with 4.5 % isoflurane gas via an induction chamber and maintained throughout surgery at 2.5 % isoflurane with a face-mask.

5.3.4.1. Critical- size calvaria bone defect for orthotropic bone formation

The surgical site was prepared by shaving and disinfecting the exposed skin. An incision of approximately 1 cm in length was made at the skull midline to expose the calvaria. A critical size defect of 3.5 mm diameter was created in each animal using a trephine drill (Helmut Zepf, Germany). The surgical procedures were performed under constant saline irrigation and care was taken to prevent injury to the dura mater. Four different conditions were tested: GNF-BMP-2 (100 µl GNF containing 1 µg of BMP-2), liquid BMP-2 (1 µg of BMP-2 in PBS 0.1 M), GNF (100 µl) alone, and an empty defect. After treating the defect as per the test conditions, the skin incision was closed with resorbable suture material (Ethicon 4.0). Six mice per condition were used. At the end of the study (8 weeks), animals were euthanized and the calvaria was harvested, fixed in 4% PFA overnight and transferred into 70% ethanol.

5.3.4.2. Subcutaneous injection for ectopic bone formation

The surgical site was prepared by shaving and disinfecting the exposed skin with betadine. Three different conditions were tested: GNF-BMP-2 (100 µl GNF containing 1 µg of BMP-2), liquid BMP-2 (1 µg of BMP-2 in PBS 0.1 M) and GNF (100 µl) alone. One hundred µl of each preparation were injected subcutaneously using a 1ml syringe with an 18-gauge needle (Terumo Europe, Belgium). Each mouse received two bilateral injections and 4 mice were used for each condition.

5.3.5. In-vivo data analysis

5.3.5.1. In vivo release of BMP-2

The *in vivo* release kinetics of injected or implanted fluorescent BMP-2 in “GNF-BMP-2” and “liquid BMP-2” was monitored at different time intervals (0 h, 8 h, 24 h, 48 h, 72 h, 120 h (day 5), 168 h (day 7), 240 h (day 10), 336 h (day 14)) using an *in vivo* photon imaging system (Lumina LT, Perkin-Elmer, USA). Mice were transferred to the “VIVOPTIC” imaging platform (agreement A33-063-919). They were anesthetized with 4.5 % isoflurane gas in an induction chamber and maintained throughout the procedure at 2.5 % with a face-mask inside the imager (TEM, France). The signal was quantified by the “Living Image” ® software. Raw fluorescence data were expressed in arbitrary units. Data were expressed as the percentage of initial mean fluorescence measured 1 hour after suturing and plotted against time. This longitudinal study was carried out on 6

animals for each group at each time point and results were expressed as an average ± standard deviation.

5.3.5.2. Planar X-Rays

After sacrifice (8 weeks), radiographic analysis of the animal back subcutis and of the 24 calvarial specimens (4 conditions, n=6) was performed using a digital X-ray imaging instrument (Faxitron X-Ray MX20-DC2, Faxitron Bioptics, Arizona, USA) at 18kV for 10 seconds. Percent radiographic bone regeneration was assessed using ImageJ® software for each calvarial specimen. A binary thresholding was performed for each radiographic image (white corresponding to new bone, black corresponding to the lack of bone). The amount of new bone was plotted against the average regeneration of the negative control (empty defect) and was expressed as an average ± standard deviation.

5.3.5.3. MicroCT (µCT) analysis

To assess mineralization, ectopic tissue explants containing the hydrogel and calvarial defects were evaluated by µCT analysis. Briefly, after reconstruction of the region of interest, bone volume (BV), corresponding to the volume of newly formed bone, and tissue mineral content (TMC), corresponding to the amount of mineral deposited within the defect, were measured. The X-Ray microtomographic device used in this study was a Quantum FX Caliper (Life Sciences, Perkin Elmer, Waltham, USA). The X-ray source was set at 90 V and 160 µA. Acquisitions were made with a 10-mm field of view. Samples were imaged with a 3D isotropic voxel size of 20 µm. Full 3D high-resolution raw data were obtained by rotating both the X-ray source and the flat panel detector 360° around the sample, with a rotation step of 0.1°. The corresponding 3600 image projections were then automatically reconstructed using RigakuSW software (Caliper, USA) into a Dicom stack of 512 files using standard back-projection techniques. The three-dimensional (3D) images of samples were built by stacking 512 cross-sections obtained by X-ray microtomography. The resulting 3D files were composed of grey-level images where the lowest grey/dark pixels represented empty spaces and the highest grey/bright pixels stood for the densest materials. Three-dimensional analyses were performed using eXploreMicroView® software (General Electric Healthcare, Milwaukee, USA). Results were expressed as an average ± standard deviation.

5.3.5.4. Histological analysis

After planar X-rays and micro-CT analysis, skin explants and calvaria were decalcified with EDTA-based Microdec® decalcifiant at pH 7.4 for 20 hours. The decalcified samples were rinsed with PBS (0.1 M) and dehydrated with graded ethanol solution finally infiltrated with toluene before embedding in paraffin. Paraffin blocks were cut as 7 µm-thick sections deposited onto Superfrost blades. These sections were de-paraffinized

and stained with Masson's trichome[411].The samples were observed using a Nanozoomer 2.0 HT slide scanner coupled to a TDI 3-CCD camera and Nanozoomer Digital Pathology software solution® (Hamamatsu Photonics, Japan).

5.3.6. Statistical analysis

All statistical analysis was performed using Graphpad Prism® software. For *in vitro* studies, measurements were performed on six different wells per condition. For *in vivo* measurement of fluorescence and bone regeneration, six mice received each treatment (24 mice in total). Assessment of statistical significance of differences was performed using ordinary one-way ANOVA for *in vitro* ALP activity and *in vivo* bone analysis. The Mann-Whitney test was used for *in vivo* fluorescent signal comparison.

5.4. Results

5.4.1. In vitro release of GNF-embedded BMP-2.

The aim of embedding the BMP-2 in the GNF was to obtain a controlled release pattern over time. The amount of released BMP-2 was measured quantitatively by ELISA and fluorescence. The fluorescence method was used to monitor the *in vivo* release of the protein in a non-invasive manner. We first tested this technique to establish whether it was a reliable tool to monitor the *in vitro* release of the gel-embedded protein. For both methods, the cumulated released BMP-2 in the supernatant was calculated as a percent (%) of the gel-loaded quantity (100 %). In the ELISA method, 100% corresponded to 1 µg BMP-2. In the fluorescence method, 100% corresponded to the mean of the initial fluorescence signals measured in the BMP-2-containing gel pieces ("GNF-BMP-2"). As shown in **Fig 24**, both methods revealed an initial burst release of BMP-2, 25% by ELISA assay and 42% by fluorescence analysis. The ELISA method showed a gradual, linear release between days 3 (72 h) and 14 (336 h) while the fluorescence assay showed a stabilization of the amount of BMP-2 released in the medium after 3 days.

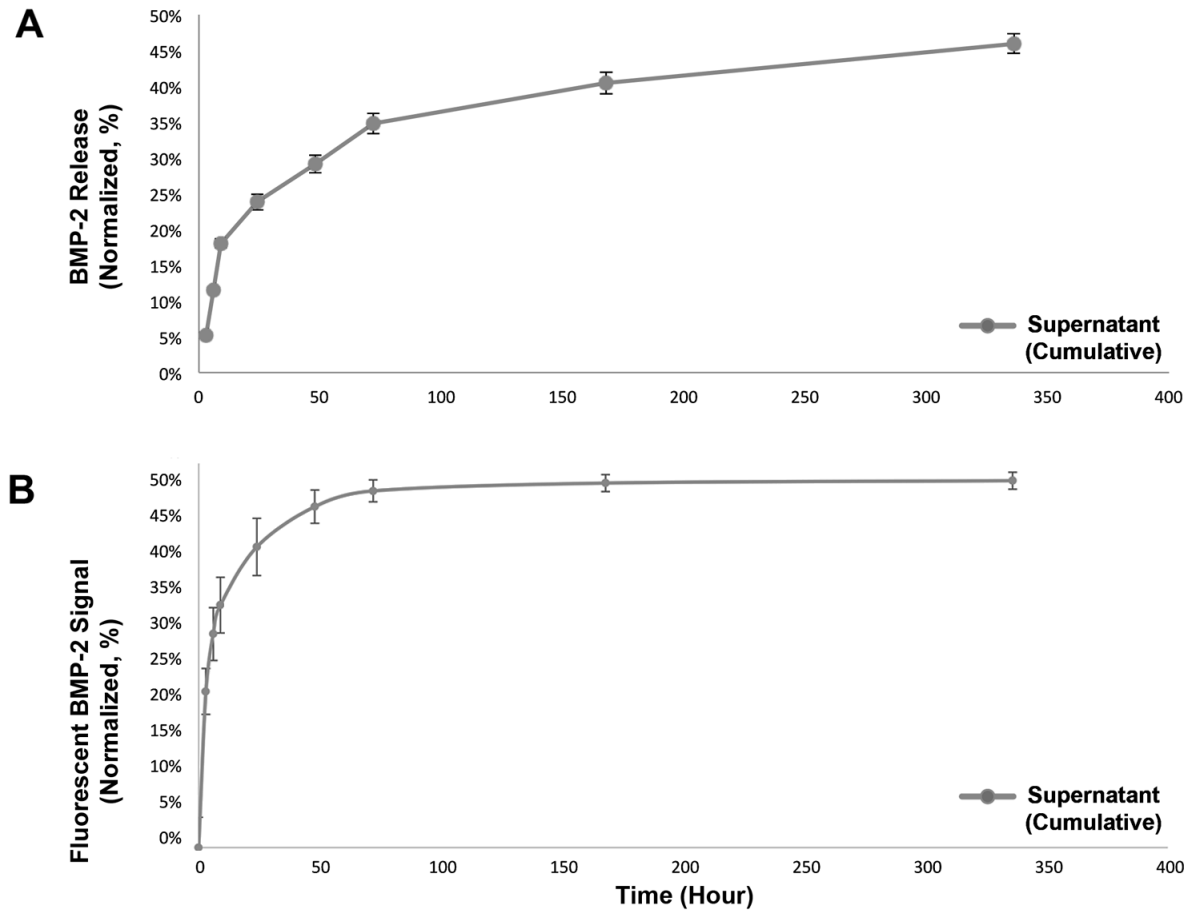

Fig.24 Time course of in-vitro release profile of GNF BMP-2

(A) BMP-2 release profile from GNF hydrogel scaffold (ELISA);(B) Fluorescent BMP-2 signal released in supernatant by GNF hydrogel scaffolds assessed by retention assay

5.4.2. Assessment of the functionality of the released BMP-2

To assess the biological activity of the BMP-2 released from the GNF hydrogel, 100 µl "GNF-BMP-2" blocks were incubated with human adipose tissue-derived stem cells (ASCs) grown on plastic tissue culture dishes. ASCs were used because they can differentiate towards the osteoblast lineage, albeit less efficiently, than bone marrow stromal cells, which require a strong and sustained osteogenic signal. The GNF gels alone did not show any positive staining for ALP activity, whereas when the gel pieces were loaded with BMP-2 (the "GNF-BMP-2"), most of the cells stained positively for ALP, as did the cells grown in the presence of osteogenic culture medium (**figure 25A, B**). Quantification of ALP activity showed a marked stimulation by gel-embedded BMP-2 (**figure 25C**), although this stimulation was less efficient than in the presence of an osteogenic medium.

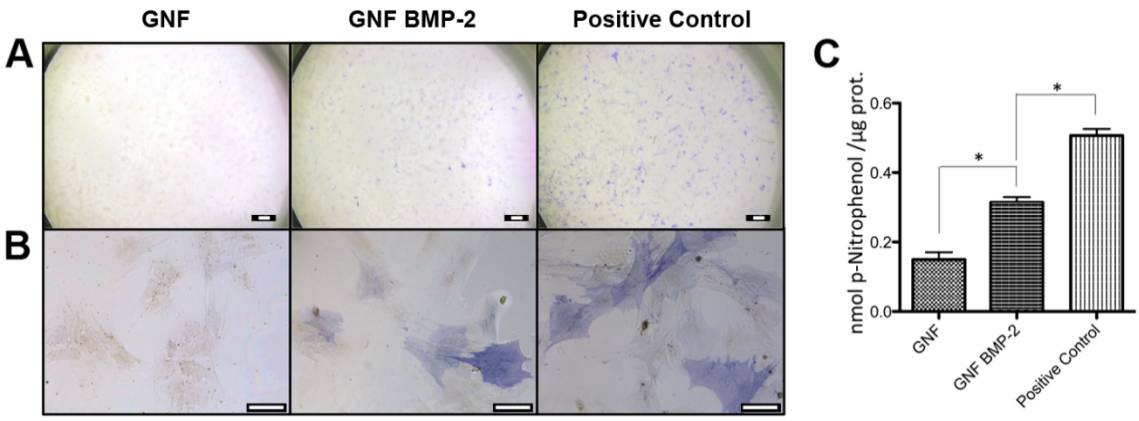

Fig.25 Effect of GNF BMP-2 on ALP activity

(A) and (B) ALP staining at day 7, scale bars are respectively 500µm and 100µm; (C) ALP quantitation at day 7(P<0.05)*

5.4.3. Analysis of bone regeneration

To determine the benefit of the GNF gel as an *in vivo* delivery system for bone repair, we used a calvarial defect in a mouse. We first evaluated the evolution of BMP-2 content within the defect. For this, we either deposited 20 µl of a BMP-2 solution or implanted 50 µl of GNF gel-embedded BMP-2 onto the 3.5-mm diameter calvarial defect of OF-1 mice. We evaluated the persistence of BMP-2 by measuring the fluorescence of tagged BMP-2. **Fig. 26** shows typical images of fluorescent signals from either “liquid BMP-2” or “GNF-BMP-2”. Quantification of the fluorescence shows that the signal measured in mice implanted with “GNF-BMP-2” was higher than that obtained from mice receiving “liquid BMP-2” (**Fig. 26**) at all time points. The difference became highly significant with time. At 14 days, the signal decreased to less than 2% of the initial signal in mice with liquid BMP-2, but still reached 8.5% of this signal with “GNF-BMP-2”. This difference suggests that the GNF gel delayed the diffusion of the protein and extended its persistence within the defect.

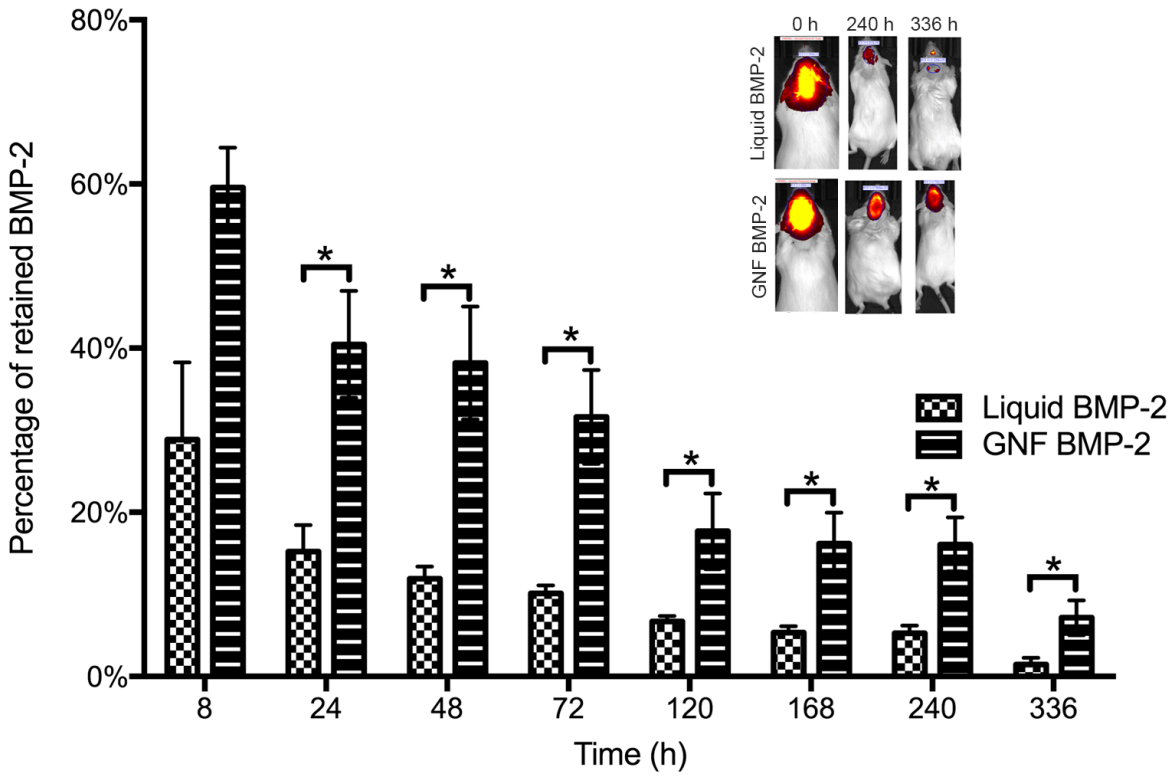

Fig.26 Time course of in-vivo release profile of GNF BMP-2 and BMP2 alone

*Fluorescent BMP-2 signal persistence in bone lesion up to 2 weeks (336 h), relative to time 0. (*P<0.05)*

Bone repair was analyzed in four different conditions: 1) in the absence of any additive or material ("empty"); 2) in the presence of protein-free GNF gel pieces ("GNF gel"); 3) in the presence of a BMP-2 solution ("Liquid BMP-2"); and 4) in the presence of GNF-embedded BMP-2 ("GNF-BMP-2"). Bone regeneration was analyzed 8 weeks after surgery. As illustrated in **figure 27A**, the "empty" and "GNF" groups did not show any significant bone regeneration. Quantitative analysis showed that in the presence of empty gels, only 10% of the lesion area was filled (**figure 28A**), whereas this percentage reached 30% with liquid BMP-2 and 62% with GNF-BMP-2. To gain insight into the 3D quantification of the newly formed bone, we measured bone volume (BV) and tissue mineral content (TMC) from 3D images obtained by micro-CT imaging. These data showed that BV (**figure 28B**) and TMC (**figure 28C**) were not significantly higher in the presence of liquid BMP-2 as compared with empty GNF gel. In contrast, GNF-BMP-2 elicited a much higher bone formation as testified by an 8-fold increase in BV and TMC as compared with protein-free GNF. These data suggest that although some bone formation had occurred in the "liquid BMP-2" group, the newly formed bone was much more abundant in the gel-embedded BMP-2. This very significant difference was essentially due to a thicker bone tissue in the latter group, as shown by lateral views of µCT images (**figure 27B**) and by histological observation of cross-sections of the calvaria

samples (**figure 27C**). Histological analyses also showed a thin layer of green-stained fibrous tissue in defects with “liquid BMP-2” or “GNF”, whereas in “GNF-BMP-2” samples, thicker, red-stained tissue was observed in the defect.

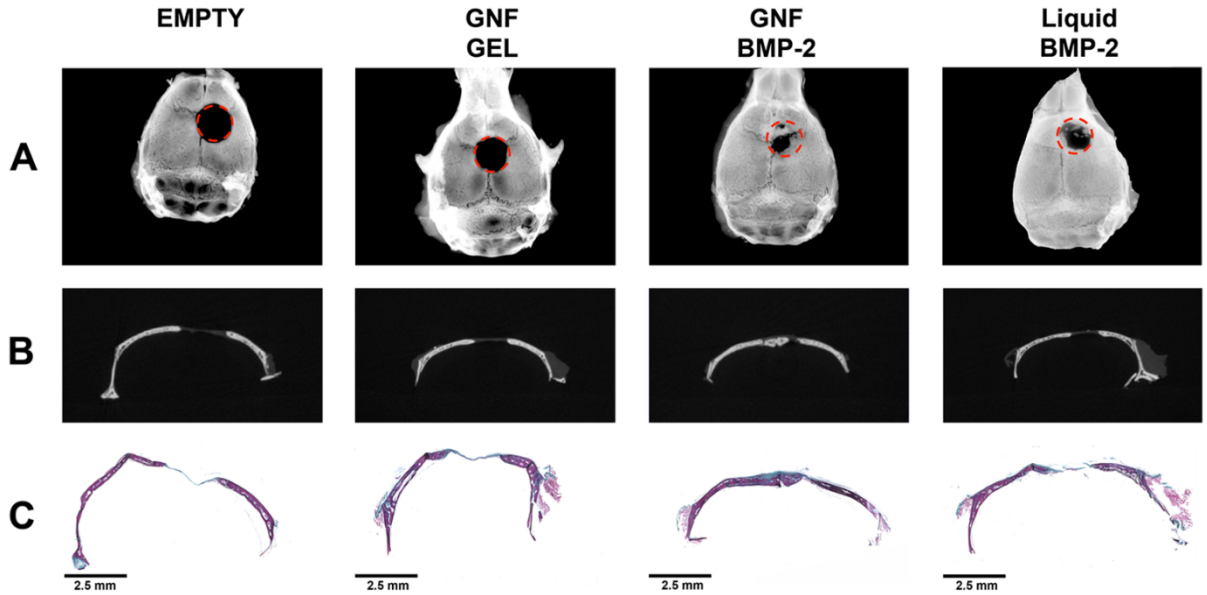

Fig.27 Effect of GNF BMP-2 hydrogels on orthotropic bone formation

Representative calvarial bone defect for each condition: (A) Planar X-ray; (B) µCT and (C) Histological Masson’s Trichrome staining (coronal plane)

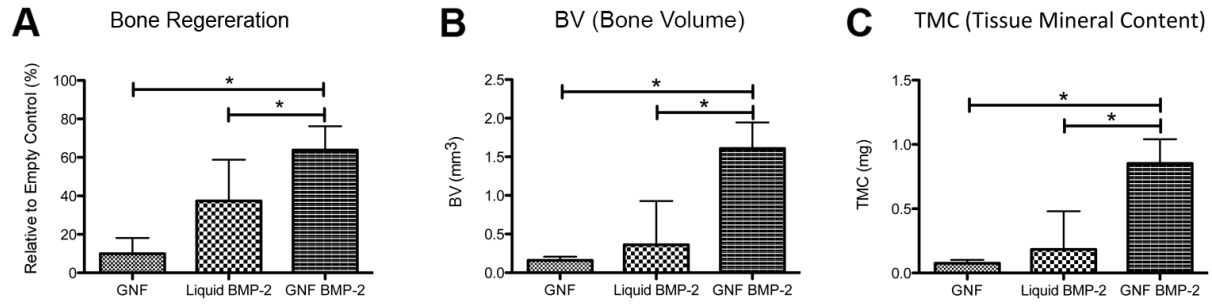

Fig.28 Computed analysis of in vivo BMP-2 bioactivity

*Quantitative analysis of calvaria bone regeneration using planar X-ray image (A), Bone Volume (B) and Tissue Mineral Content (C) using µCT analysis. *P<0.05*

Careful examination of “GNF-BMP-2” samples was carried out to obtain more insights into the qualitative analysis of the thick tissue covering the defect. **Fig. 29A** shows newly formed bone clearly identified within the defect. Unlike native bone, it was mainly formed of primary woven bone. A few small pieces of un-degraded gel were visible (black stars). Fibrous tissue, which was still predominant at the outer edge of the defect, was occasionally observed within the primary bone areas (**fig. 29B**, black arrows). Many

blood vessels were observed (green arrowheads). Pores containing bone marrow were also present (**fig. 29C**, yellow dashed line). Numerous osteocytes were found embedded in the primary bone matrix (white dash-line). Only a few areas showed a secondary ossification pattern with dense lamellar bone (red dashed line).

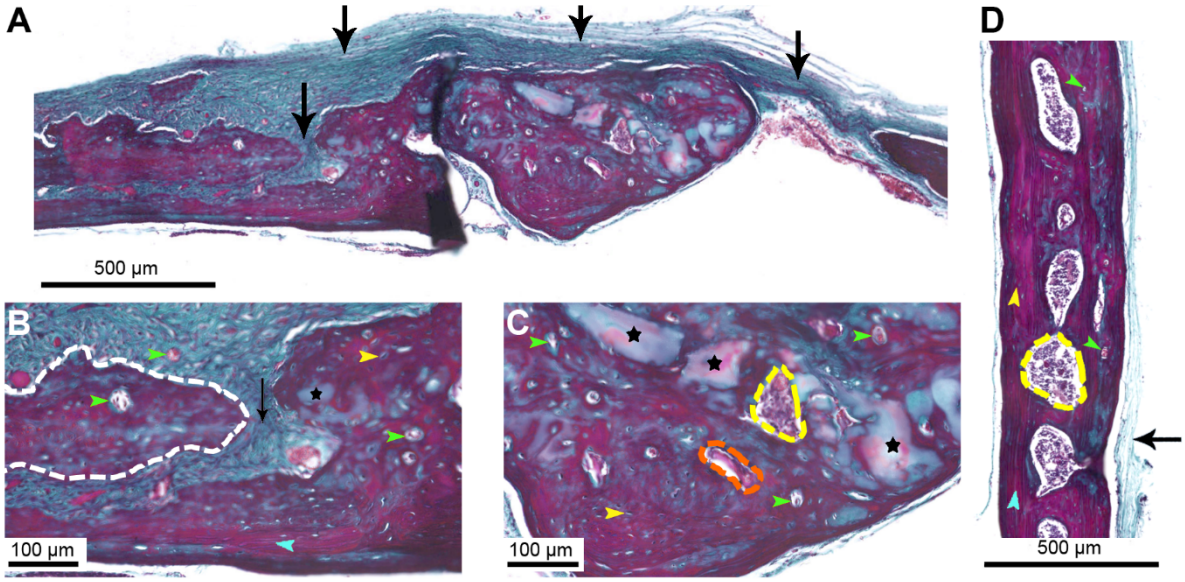

Fig.29 Histological analysis of mice calvaria implanted with GNF BMP-2 hydrogels

Black arrows show defect-filling fibrous tissue, green arrowheads mark representative vessels, yellow arrowheads mark representative osteocytes. Black stars indicate representative GNF gel blocks. White dashed line delimits primary bone in remodeling, red dashed line shows secondary ossification center, and yellow dashed line surrounds bone marrow. Magnification: (A) 5X, scale bar 500µm; (B) and (C) 20X, scale bars 100µm.

5.4.4. Analysis of ectopic bone formation

To assess the osteoinductive capacity of GNF-embedded BMP-2, we injected either "liquid BMP-2" or "GNF-BMP-2" subcutaneously in 12-week-old OF-1 female mice. One group received empty GNF ("GNF"). Monitoring of BMP-2 quantity at the injection site showed a much slower decrease in the fluorescent signal in the "GNF- BMP2" group than in the "liquid BMP2" group. Among the four mice which had received the gel-embedded BMP2, one did not show any signal at any time post-operatively. Eight weeks after injection, a significant signal was still observed in the 3 mice in the "GNF BMP2" group (**fig. 30A**), whereas it was undetectable in all the "liquid BMP2" mice. Planar X-ray acquisitions showed the presence of X-ray-opaque structures at the injection site 8 weeks after injection (**fig.30B**) only in the three mice which had received the GNF-embedded BMP2, and which emitted a fluorescence signal at that time. Upon gross examination of biopsies, empty GNF appeared as soft white pellets (data not shown). In contrast, BMP2-containing GNF appeared as hard red blocks (**fig. 30C**). Micro-

computerized X-ray tomography examination of the biopsies showed the presence of trabecular-like mineralized structures with a mineral density of 650 mg/cm3, similar to that found for mouse native trabecular bone (**fig. 30D**). The average thickness of these trabeculae was 18 µm, a value similar to that measured in the trabeculae of mouse tibia and femur.

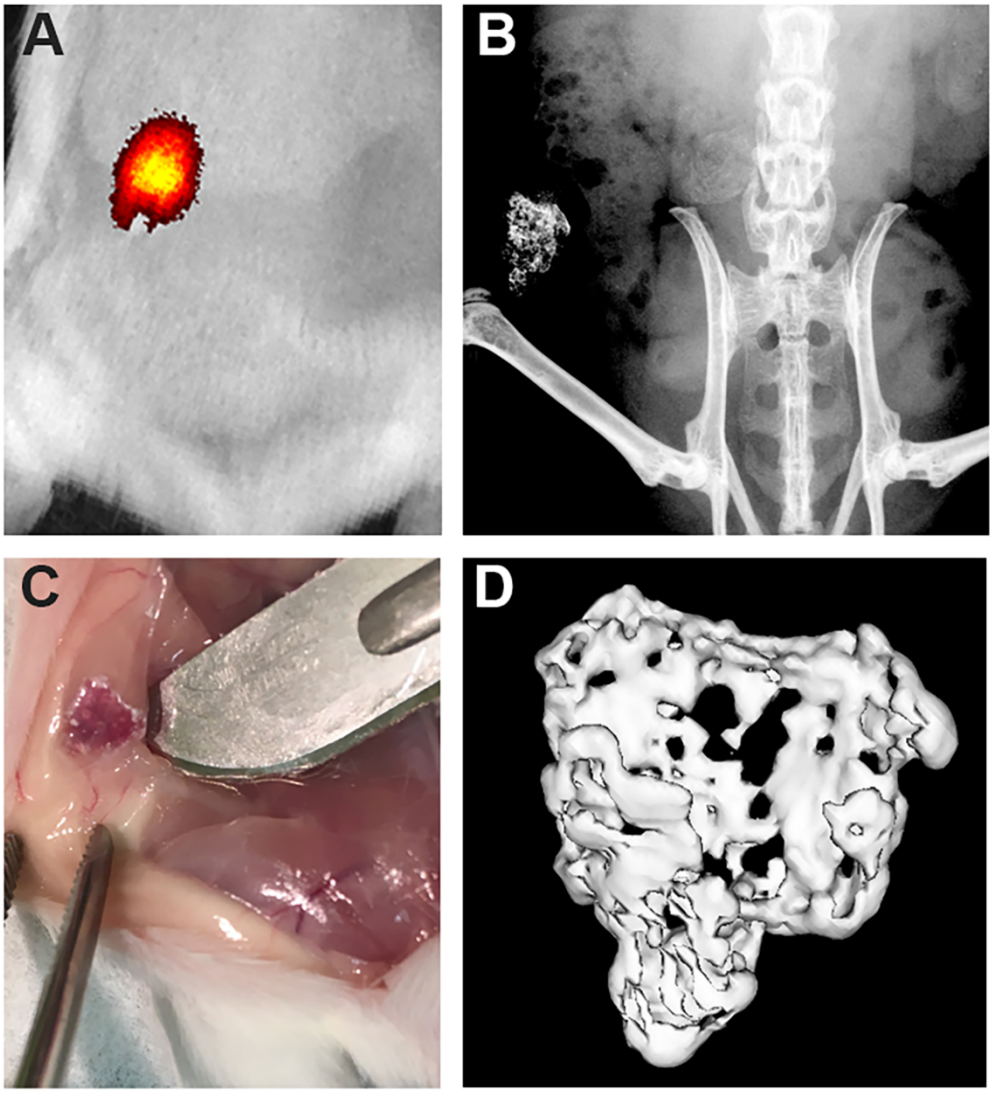

Figure 30 Effect of GNF BMP-2 hydrogels on ectopic bone formation

(A) Fluorescence signal assessed by in-vivo imaging; (B) Planar X-ray radiography 8 weeks after injection; (C) Macroscopic examination of ectopic bone in skin biopsy immediately after sacrifice; (D) Representative µ-CT image

Histological examination using Masson's trichrome staining revealed a complex interpenetrated network consisting of intact or resorbing gel pieces and bone tissue (**fig. 31 A**). In some areas, gel blocks were surrounded by inflammatory tissue mainly composed of macrophages (data not shown). However, most areas consisted of mineralized trabeculae filled with bone marrow. The density of the trabecular matrix

varied from a woven-bone-like organization (**fig. 31 C**) to mature lamellar bone (**fig. 31 B, D, E**). Lining cells and osteoclasts were observed on the surface of these bone structures (**fig.31 B**). Many osteocytes were found within the bone matrix. The marrow was characterized by the presence of numerous erythrocytes, the presence of other cells of the hematopoietic lineage such as eosinophils and megakaryocytes, and many small blood vessels. Gel pieces were scattered within the marrow and trabeculae. Importantly, bone matrix formation was observed at the surface of many gel pieces (**fig. 31 D, E**), together with the presence of osteoblasts. Moreover, bone matrix formation was occasionally observed as thin trabeculae penetrating the gel blocks (figure 7E).

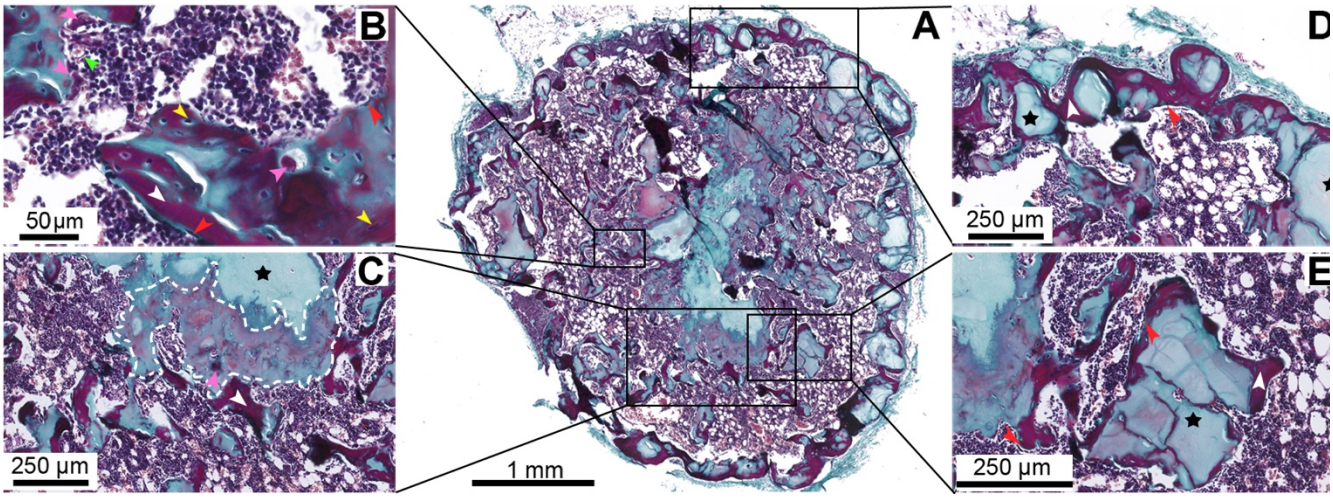

Figure 31 Fig. 8 Histological analysis of subcutaneous explanted GNF BMP-2 hydrogels

Black stars mark representative GNF gel blocks. Green arrowheads mark vessels, white arrowheads indicate representative lamellar bone tissue, pink arrowheads show representative osteoclasts, yellow arrowheads indicate representative osteocytes, pink arrowheads show representative osteoclasts and red arrowheads point to representative bone lining cells. White dashed line delimits primary bone in remodeling, red dashed line shows secondary ossification center, and yellow dashed line indicates bone marrow. Magnification: (A) 2X, scale bar 1 mm; (B) 40X, scale bars 50µm; (C) and (D) 10X, scale bars 250µm; (E) 15X, scale bars 250µm.

5.5. Discussion

Bone morphogenetic protein 2 (BMP-2) is used in a few clinical applications for bone regeneration therapies[412,413]. However, the therapeutic applications could become more numerous if its adverse effects could be overcome[414,415]. Indeed, there is compelling evidence that in the absence of an appropriate delivery system, its side-effects outweigh its therapeutic advantages.

In the study reported here, we have designed a new carrier system for BMP-2 delivery based on a non-polymer hydrogel that rapidly gels at physiological temperature. The relatively slow kinetics of *in vitro* gel formation in a physiological medium upon cooling allows easy encapsulation of fragile molecules like proteins. No complex chemical procedures are needed to form the gel or to encapsulate the protein, obviating the need for extensive washing or dialysis procedures. Instead, the straightforward procedure used to encapsulate the protein in the GNF hydrogel, based on a simple physical gel that forms spontaneously within a few minutes just prior to gel injection or implantation, is compatible with extemporaneous preparation and easy on-site handling by medical staff.

Its *in vitro* release kinetics was established by conventional ELISA assay and, in parallel, by fluorescence measurement of tagged BMP2. The aim of using a tagged protein was to establish a method enabling the monitoring of the release kinetics *in vivo*. The *in vitro* assay showed that the measured fluorescence did not perfectly match the curve obtained by the standard method. The burst release appeared more pronounced with the fluorescence method. In addition, the curve reached a plateau whereas a slow linear slope was observed with the ELISA method. This difference may be due to the lower sensitivity of the fluorescence assay. Indeed, the signal emitted by the supernatants became close to the sensitivity threshold with time. The dilution of the released protein in a relatively large volume of buffer may account for this low signal. For the *in vitro* release assay, the ELISA method clearly provides the most reliable data. We hypothesize that the burst release results from syneresis, a physical phenomenon that consists of shrinkage and fluid release and which occurs following the formation of the GNF-based gel as well as with several other hydrogels[416]. The second slower phase might result from the diffusion of BMP-2 from the gel. The chemical composition of these gels does not suggest any possible interaction with BMP-2 since the GNF molecule does not present any charge or motif with a potential affinity for the protein. The structure of the GNF-based hydrogel has been shown to consist of interconnected fibers[121] that are thought to be made of micelles with a hydrophobic core and an external hydrophilic moiety composed of the glucose and thymidine residues[120]. The dense network

formed by these micelles might thus slow down the diffusion of the protein, thereby ascribing GNF-based hydrogels to the category of delivery systems based on simple physical encapsulation and diffusion-based release[73]. An alternative to simple diffusion is degradation-dependent release. This hypothesis is supported by the slow degradation rate of GNF-based gels observed *in vitro* and *in vivo*[123]. Previous studies have shown that gels made of GNF are cytocompatible[121]. In the present study, incubation of GNF gel pieces with primary hASCs did not elicit cell death (data not shown). “GNF-BMP-2” stimulated the *in vitro* differentiation of hASCs, showing that the protein was released from the gel and was biologically active. Although the monitoring of BMP-2 release by the fluorescence technique may not be appropriate for the precise quantification of the protein, we believed that it would provide useful information about the diffusion of BMP2. Therefore, we considered it appropriate for assessing the fate of *in vivo*-administered BMP-2. In both the subcutaneous and the calvarial defect models, we observed a relatively fast decrease in the “liquid BMP-2” group. Eight weeks after injection (subcutaneous) or implantation (calvaria), the signal was either undetectable or close to the sensitivity threshold, respectively. The “GNF-BMP2” groups showed a significantly higher signal and this difference between the two groups was greater in the subcutaneous model. These data show that GNF embedding enables the gradual release of BMP-2. The pharmacokinetics of “GNF-BMP-2” is comparable to that observed in other studies where ^{125}I-tagged BMP-2 was used as a tracer to monitor the persistence of calcium phosphate ceramics[417], collagen sponge-embedded[418] or gelatin-embedded[419] protein at the site of implantation. Our data using fluorescent tagged BMP2 provide useful information about its pharmacokinetics after its *in vivo* administration, but this very simple technique is limited to use at superficial anatomical sites.

Since we were able to monitor BMP-2 *in vivo*, we could establish a correlation between the long-term persistence of the protein and its capacity to induce bone formation in two different models. Strikingly, in the subcutaneous model, there was a close correlation between the persistence of BMP2 at 8 weeks and deposition of a mineralized matrix within the GNF gel. In the only mouse which had received the GNF-BMP2 but failed to show a fluorescent signal, no mineralized structures were observed. In the calvarial defect, there was a limited bone formation when liquid BMP2 was administered, correlating with a rapidly decreasing fluorescence signal. When gel-embedded BMP2 was deposited, a much higher amount of bone was formed, again correlating with a high fluorescence signal. Indeed, the assessment of the remanence of BMP-2 and its correlation with bone formation would seem to be a powerful method to demonstrate the

benefit of the GNF gel as a carrier that delays and sustains the release of the active growth factor.

These data show the high osteoinductive capacity of the GNF-BMP-2 system and highlight the unique properties of the GNF gel to promote the formation of high-quality bone. In the calvarial bone defect model, GNF-BMP-2 increased the reconstructed bone surface and the thickness of the regenerated bone tissue, whereas liquid BMP-2 resulted in less complete filling of the defect by thin irregular mineralized tissue. Moreover, qualitative analysis of the newly formed tissue revealed typical features of actively remodeling bone. Primary woven bone invaded by blood vessels still contained a few pieces of GNF gel. A few nuclei of secondary ossification centers were observed, as well as bone marrow islets. These observations suggest that GNF-BMP-2 promotes the progressive regeneration of physiological bone tissue with all the characteristics of the original native calvarial tissue. The persistence of a few gel fragments suggests that the gel degradation kinetics is well-fitted to the regeneration process. Moreover, the progressivity of the repair process may favor the reconstruction of a fully mature bone, identical to the native calvarial bone. In summary, GNF-BMP-2 promoted the formation of new calvarial bone while the gel underwent gradual degradation. This robust osteogenic activity of GNF-embedded BMP-2 is similar to that previously described for other BMP2-laden carriers in the calvaria bone repair model[240,420–431].

However, our data also show how this system can elicit efficient bone formation in the subcutaneous injection model. The product is injectable and retains its original size, shape (between a cylinder and a parallelepiped) and position at the site of injection. In fact, it has outstanding osteogenic potential. The structures formed 8 weeks after injection are very similar to native trabecular bone, showing an abundant bone marrow interspersed with mineralized trabeculae. Thus, GNF seems perfectly adapted to bone regeneration, with a degradation kinetics well correlated with new bone formation. Moreover, GNF blocks were infiltrated by bone-forming cells and bone deposits were observed on their surface, suggesting that GNF is osteoconductive and osteogenic. These observations are in agreement with our previous report of the osteogenic capacity of GNF in the presence of human stromal cells[123]. Together, these observations raise the enticing possibility of creating fully functional remodeled trabecular bone. Although previous studies have demonstrated the bone formation activity of BMP-2 in subcutaneous implantation models[337,400,419,432–441], this is the first time to our knowledge that an injectable, gel-based BMP2 delivery system has resulted in the formation of highly structured bone in this model. Previous studies demonstrated bone

formation by an injected hyaluronan gel, but the bone was observed only at the periphery of the injected gel[442]. This new osteogenic potency, with bone forming within and around the injected gel, may result from the original properties of GNF gels and especially from its intrinsic osteogenic potential.

In conclusion, this study shows that GNF gel is an efficient, easy-to-handle delivery system for bone regeneration applications. It also shows that GNF gels can be used as a template scaffold for the regeneration of bone tissue.

Acknowledgment

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6. Discussion générale

Dans les domaines de l'orthopédie, de la chirurgie buccale et maxillo-faciale, la régénération osseuse reste un défi clinique. Le traitement standard actuel est basé sur l'utilisation de matériaux qui sont divisés en deux groupes majeurs: greffes osseuses naturelles et synthétiques[443]. Les greffes osseuses naturelles comprennent les auto-/allo- et les xénogreffes. Le greffon osseux autologue est considéré comme le traitement standard pour la réparation et la régénération osseuse par de nombreux chirurgiens, principalement en raison de l'absence de réaction immunogène et d'une performance biologique optimale en termes d'ostéogénicité, d'ostéoinductivité et d'ostéoconductivité[151]. Cependant, ces greffes osseuses présentent plusieurs limitations : une disponibilité limitée, la nécessité d'une procédure chirurgicale supplémentaire pour le prélèvement, la morbidité du site du donneur, la douleur post-opératoire et les risques d'infection[56]. Les allogreffes et les xénogreffes sont quant à elles largement disponibles et il n'est pas nécessaire d'effectuer une chirurgie supplémentaire. Cependant, avec ces types de greffes il existe un risque d'immunoréaction et leurs potentiels ostéoinductifs et ostéoconducteurs sont réduits à cause des traitements que doivent subir les greffons[151,444]. Pour surmonter ces limites, un grand nombre de matériaux et de stratégies ont été développés et étudiés au cours des dernières décennies. Ces matériaux présentent plusieurs avantages : une large disponibilité, l'absence de réponse antigénique et la possibilité de fonctionnalisation pour une application spécifique. Cependant, à l'heure actuelle il n'existe pas de matériau idéal pour l'ITO. Tout d'abord, puisque comme il a été décrit il n'existe pas un mais plusieurs types de lésions osseuses qui auront des localisations, des tailles et des origines différentes. De plus la qualité des tissus autour de la lésion est à prendre en considération. Il a été montré qu'il existe de nombreux hydrogels qui offrent des propriétés ostéoinductives ou ostéoconductives en présence de cellules exogènes ou de facteurs de croissance[211,224,228,232]. Cependant, ces mêmes matériaux peuvent être mal adaptés à certaines applications cliniques sans avoir recours à de complexes modifications, parfois leurs propriétés confèrent au matériau une faible stabilité mécanique (cas des hydrogels à base de peptides sans modifications)[235] ou encore entrainent une dégradation trop rapide lorsqu'ils sont implantés (cas des polyphosphoesters)[232].

Au cours de ces travaux nous avons développé de nouvelles stratégies d'ITO entièrement axées sur l'utilisation d'un composé récemment synthétisé. Ainsi nous avons étudié l'apport d'un hydrogel physique dans une stratégie de régénération par

l'apport de cellules exogènes puis dans une stratégie de libération de facteurs de croissance.

6.1. L'hydrogel de GNF

Nos travaux de recherche se sont basés sur l'utilisation d'un hydrogel physique généré par l'auto-assemblage de monomère de GNF pour l'ITO[121]. Cet hydrogel a précédemment été décrit comme étant biocompatible, bio intégrable et biodégradable [123]. Ce matériau est un hydrogel non-polymérique. Ainsi son originalité se traduit par l'absence de procédures chimiques complexes pour initier sa gélification contrairement à d'autres hydrogels comme certains polymères synthétiques qui contiennent des agents réticulant toxiques[134]. En effet, les étapes de formation du matériau ne nécessitent pas de réticulation par des agents chimiques, ni de lavages répétés ou de dialyses et limitent considérablement le risque de libération de composés toxiques lors de l'implantation du matériau. Il est à noter que la composition du GNF ne comporte aucune liaison chimique clivable par les enzymes produites par les tissus humains. Enfin, les propriétés de ce matériau font qu'il gélifie en quelques minutes (20-30 minutes) à température physiologique, ce qui permet d'y incorporer des cellules ou autres protéines sans risque de choc thermique avant d'être injecté au sein du site lésé. Néanmoins cet hydrogel ne dispose pas de motifs permettant l'adhésion cellulaire (via les intégrines par exemple).

6.2. La conception d'un hydrogel composite

Comme il a été décrit dans la revue de la littérature, de nombreuses études récentes ont souligné l'intérêt des approches où des hydrogels synthétiques sont associés à des hydrogels naturels pour leurs propriétés biologiques[219]. L'objectif de ces approches est de surmonter les limites de chacun des composés grâce aux propriétés complémentaires du matériau avec lequel ils sont associés. C'est dans ce contexte qu'une partie de notre travail de recherche a été d'associer le gel de GNF à un hydrogel de collagène. Le collagène, composant majeur de la MEC, est utilisé en tant que biomatériau naturel, il est biodégradable et biocompatible ce qui en fait un choix évident pour le développement de matrices pour des applications en ingénierie tissulaire[107,382]. Néanmoins, les hydrogels de collagène présentent des propriétés mécaniques faibles et des taux de dégradation rapides, que ce soit *in vitro* ou *in vivo*[155,383]. Nos travaux ont confirmé ces limitations puisque le module élastique de ces hydrogels (rhéologie) est de 10 Pa.

Conformément aux études précédentes[387,388], nous avons également observé une importante contraction des hydrogels de collagène lorsqu'ils sont ensemencés de cellules (hASCs). Il en résulte une altération de la survie cellulaire due à la réduction de la diffusion des éléments nutritifs au sein du matériau et une fuite des cellules hors de celui-ci liés aux faibles propriétés mécaniques des hydrogels de collagène. Ainsi notre objectif a été de combiner les propriétés mécaniques plus fortes (1 kPa) et ostéoinductives du gel de GNF[123] avec les excellentes propriétés d'adhésion cellulaire du gel de collagène[107].

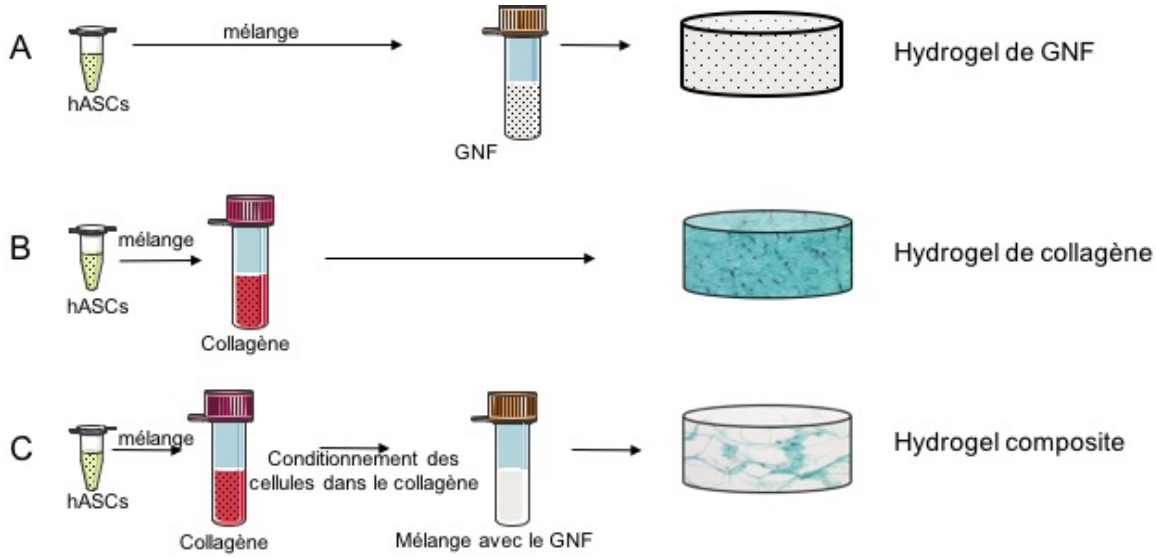

Figure 33 Méthodes de préparation des hydrogels

L'étude des propriétés de ce matériau composite a montré que les propriétés rhéologiques sont largement améliorées lorsque le gel de collagène est associé à un autre matériau, ce qui corrobore les résultats présentés dans la revue de la littérature[389],[390]. Ceci s'est traduit par une limitation signification de la perte de volume des matériaux que ce soit *in vitro* après leur formation ou *in vivo* après leur injection. D'autres auteurs se sont intéressés à ce type de stratégies et sont parvenu à améliorer les propriétés mécaniques du collagène par l'ajout d'un hydrogel synthétique [251] pour guider la différenciation cellulaire. Cependant ces stratégies font recours à des matériaux qui sont non-biodégradable et peuvent entrainer une certaine cytotoxicité[250].

Concernant les propriétés biologiques, la survie des cellules et leur prolifération a été observée *in vitro* et *in vivo* au sein du matériau composite. Ceci souligne l'importance des propriétés biologiques du gel de collagène dans le matériau composite. Cette étude a révélé une autre propriété très prometteuse du gel composite, les cellules

ensemencées au sein du matériau ont révélé leur capacité à se différencier en cellules de phénotype ostéoblastique *in vitro* ainsi qu'à former des structures pseudo-osseuses *in vivo*. Cette capacité du gel de GNF à promouvoir la différenciation en cellules osseuse avait été précédemment observée lors des travaux de thèse de S. Ziane, il a également été décrit dans la littérature que le collagène est capable de promouvoir cette différenciation ostéoblastique [393]. Il n'est donc pas possible de déterminer lequel des composants est responsable de ce phénomène ni s'il s'agit d'un effet de synergie. Cette étude fait ainsi apparaitre ce matériau composite comme un candidat prometteur pour l'ITO comme échafaudage pour des cellules humaines, de par ses propriétés ostéogéniques où le collagène offre une matrice pour l'adhésion cellulaire et le GNF apporte un soutien mécanique à la structure.

6.3. Le choix du type cellulaire

Si le choix et la conception du meilleur échafaudage pour l'ITO est complexe, il en est de même pour le choix de la source de cellules et les techniques de préparation et de pré-conditionnement de celles-ci[128,329]. Ici, nous avons choisi d'utiliser des cellules souches dérivées du tissu adipeux, une source cellulaire très attrayante pour la médecine régénératrice notamment car elles sont techniquement faciles à collecter[445]. Il est facile d'en obtenir un nombre élevé par rapport aux cellules souches issues de la moelle osseuse et comme il a été décrit précédemment elles ont la capacité de se différencier en plusieurs types cellulaires, dont les ostéoblastes[94,446,447]. Cependant les stratégies reposant sur l'association de cellules souches exogènes à un matériau échafaudage présentent certaines limitations comme le coût élevé de ces procédures[448], la gestion logistique (clinique-laboratoire) ou le risque d'altération génétique des cellules lié aux processus de culture prolongé lors des phases d'amplification par exemple[449]

6.4. L'utilisation d'un facteur de croissance

Afin de s'affranchir des contraintes inhérentes à l'utilisation des cellules souches exogènes, nous nous sommes intéressés au développement d'une deuxième stratégie d'ingénierie tissulaire. Cette stratégie a reposé sur l'utilisation d'un facteur de croissance approprié à l'ITO et pouvant être libéré de façon progressive grâce à l'utilisation d'un hydrogel. Ainsi, nous avons associé la BMP-2, facteur de croissance ostéoinducteur utilisé en clinique[126], avec l'hydrogel de GNF pour promouvoir la régénération osseuse grâce à l'implantation du composé au sein lésion osseuse.

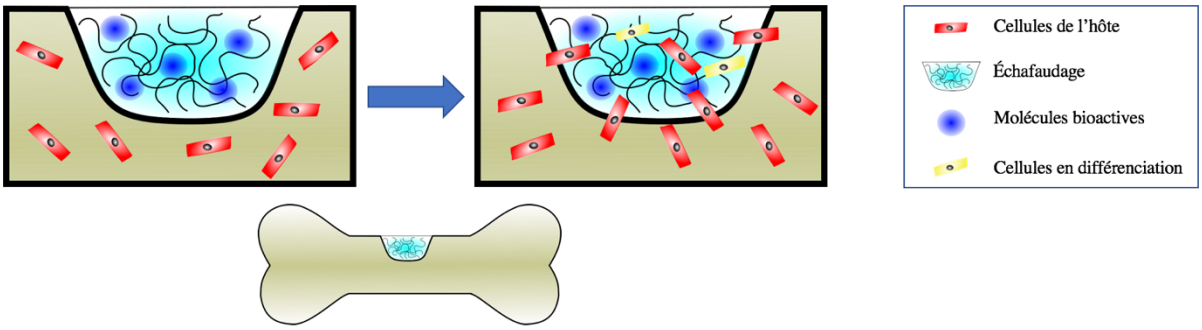

Figure 34 : Stratégie d'ITO Échafaudage pour la libération de BMP-2

L'objectif de cette stratégie a donc été de s'affranchir de l'ajout de cellules exogènes mais de promouvoir le recrutement des cellules de l'hôte et de stimuler leur différenciation, sous l'effet de la BMP-2.

Il a été montré que la structure des hydrogels de GNF est constituée de fibres interconnectées[121] qui sont composées de micelles avec un cœur hydrophobe et une partie externe hydrophile[120]. L'hypothèse a été que le réseau dense formé par ces micelles permet la diffusion lente du facteur de croissance faisant ainsi du gel de GNF un système de libération lente basé simplement sur sa capacité à « encapsuler » physiquement puis à libérer la BMP-2 comme l'ont décrit les travaux de Mooney *et al*[73]. Une observation de l'hydrogel en cryo-microscopie à balayage a permis de mettre en évidence la structure fibrillaire du matériau. Ce résultat est présenté en Annexe 2. Il est important de souligner que l'utilisation du GNF comme matériaux pour la diffusion de la BMP-2 nous a permis d'obtenir les résultats ci-avant présentés avec une dose de BMP-2 très faible : 1 µg/cc contre 1,5 mg/cc dans les protocoles habituels [126].

6.5. L'étude des propriétés ostéinductives

Après avoir validé la faisabilité et l'efficacité du système de libération contrôlé de BMP-2 *in vitro*, ce système a été injecté chez la souris en site sous-cutané.

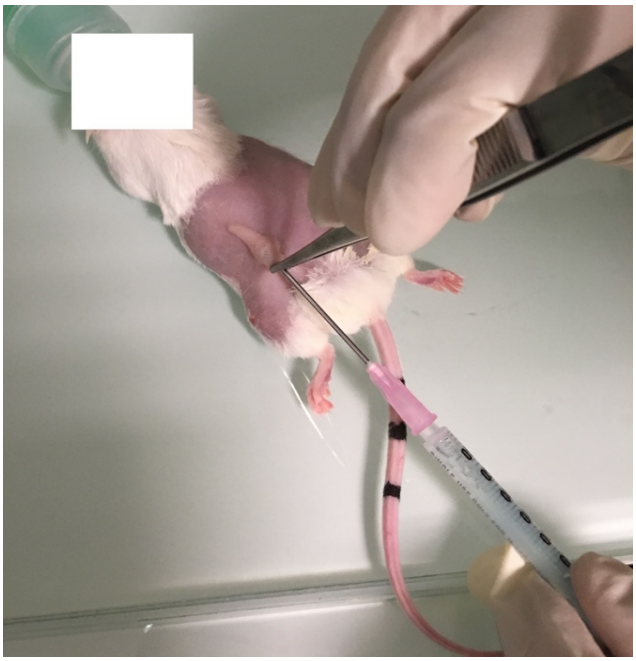

Figure 35 : Injection en site sous-cutané du complexe hydrogel chargé en BMP-2

Ces injections ont permis tout d'abord de s'assurer de la bonne injectabilité du système et de la capacité des hydrogels à retenir la BMP-2 sur le site d'injection afin qu'elle soit libérée progressivement. Une étude longitudinale d'une durée de 8 semaines a été réalisée. Ce modèle permet également d'étudier les capacités ostéoinductives de la stratégie d'ITO mise en place. Comme décrit précédemment un matériaux est ostéoinducteur lorsqu'il est capable de promouvoir l'ostéogenèse au sein de la matrice implantée, en stimulant les cellules de l'hôte, recrutées, vers une différenciation en cellules osseuses (préostéoblastes, ostéoblastes)[87]. Ainsi une injection en site sous cutané permet de s'assurer que ce phénomène ne serait pas lié à la proximité du matériau avec le tissu osseux de l'animal (dans ce cas il s'agirait d'ostéo-conduction) puisque l'injection est réalisée à distance de tout os.

6.6. Le modèle de lésion osseuse de la calvaria

Afin d'étudier les capacités du sytème à promouvoir la régénération osseuse, un modèle de lésion osseuse de taille critique de la calvaria a été réalisé chez la souris. Ce modèle a été choisi pour la manipulation aisée des souris, sa reproductibilité, son faible coût et la possibilité de réaliser un suivi post-chirurgie (suivi par fluorescence de la BMP-2 implantée)[450]. Depuis plus de 25 ans la lésion de calvaria est employée comme modèle *in vivo* pour étudier les matériaux de comblement osseux et les stratégies de régénération osseuse (comprenant les approches basées sur l'apport de cellules, de protéines ou de gènes)[451,452]. Ce modèle de lésion rend les résultats plus pertinents d'un point de vue physiologique que ceux obtenus en sites ectopiques[453].

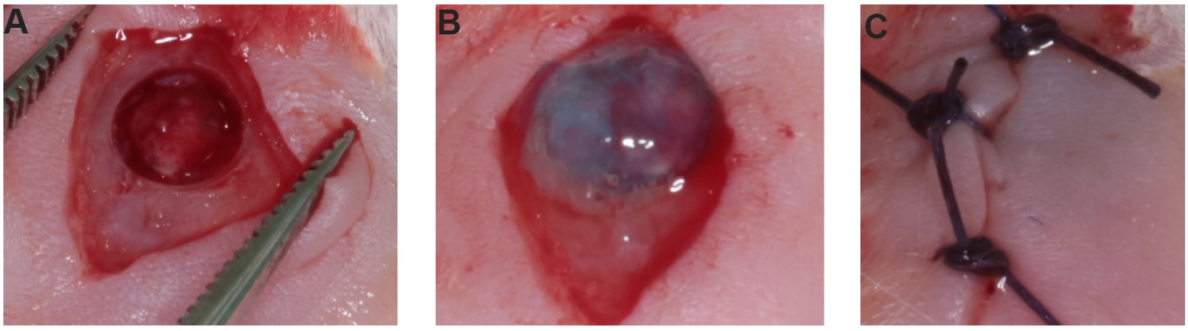

Figure 36 : (A) Réalisation d'une lésion osseuse de la calvaria chez la souris, (B) implantation de l'hydgrogel contenant la BMP-2, (C) suture de l'incision cutanée

Les résultats obtenus 8 semaines après l'implantation des échafaudages de GNF contenant la BMP-2 soulignaient une capacité de ces derniers à promouvoir la régénération osseuse avec une réparation moyenne de la lésion s'élevant à près de 70 % alors que la réparation naturelle est nulle. De plus, l'étude histologique du tissu osseux nouvellement formé a révélé des caractéristiques d'un os en cours de remodelage.

Le tissu osseux primaire qui contenait encore des fragments de gel était envahi de vaisseaux sanguins et présentaient des centres d'ossification secondaire ainsi que de la moelle osseuse. Ces observations suggèrent que le gel de GNF chargé en BMP-2 se dégrade progressivement laissant place à un tissu osseux à l'aspect proche de celui de l'os natif de la calvaria.

Les modèles *in vivo* utilisés au cours de nos travaux ont également permis de mettre en avant les propriétés ostéoconductrices et ostéogéniques de l'hydrogel de GNF. En effet les blocs de GNF ont été infiltrés par des cellules ostéo-formatrices. De plus, des dépôts de tissus minéralisés étaient observés sur la surface des gels de GNF. Ces observations concordent avec les précédentes études sur la capacité ostéogénique du GNF en présence de cellules stromales humaines[123] et font du gel de GNF un matériau prometteur pour une utilisation l'ITO.

7. Limites des modèles et des outils ; perspectives

L'objectif de ce projet a été de concevoir et d'étudier des stratégies d'ITO basées sur l'utilisation d'hydrogels pour l'injection de cellules souches ou la libération de facteurs de croissance.

7.1. L'hydrogel de GNF

Lors des études réalisées nous avons pu apprécier les qualités mécaniques du matériau. En effet les propriétés du gel de GNF permettent son injection et sa persistance au sein du site anatomique *in vivo*. Les résultats montrent également que le gel de GNF a la capacité de stabiliser mécaniquement une matrice de gel de collagène afin d'éviter les phénomènes de compaction et de perte des cellules ensemencées au sein du gel de collagène. Les propriétés du gel de GNF ne permettent pas la survie de cellules ensemencées au sein du matériau. Néanmoins, les propriétés structurelles du matériau permettent à l'hydrogel de contenir et libérer progressivement des facteurs de croissance de façon ciblée sur site osseux lésé.

Il est donc nécessaire d'associer une matrice naturelle contenant les cellules pour assurer leur adhérence, leur survie et leur prolifération, *in vitro* et *in vivo*. Pour que ce matériau offre de meilleures performances biologiques il reste des améliorations à réaliser en amont, lors du développement et de la synthèse des molécules gélatrices. Une stratégie serait le greffage de molécules d'adhésion aux matériaux comme par exemple des peptides RGD. Il a été rapporté que des stratégies comme le greffage de motifs RGD de façon contrôlée à l'hydrogel favorise l'adhésion, la survie et la différenciation des cellules souches ensemencées au sein de l'hydrogel[195,196]. Au cours des travaux expérimentaux menés, des résultats contradictoires ont parfois été observés en termes de comportement rhéologique et biologique (survie et différenciation cellulaire). Il s'est avéré qu'un lot de poudre nécessaire à la préparation des hydrogels contenait de nombreuses contaminations et impuretés révélées à postériori par analyse RMN. Ces contaminations peuvent être dues à un problème lors des étapes de synthèses de la molécule ou à un mauvais stockage de la poudre de GNF. Nous avons tiré des enseignements de ces expériences, lorsque l'on travaille avec un nouveau composé, pour lequel aucun protocole d'utilisation ou de conservation n'est encore validé : avant chaque expérience un contrôle qualité du produit est nécessaire.

Une autre limite causée par l'utilisation d'hydrogels a été l'impossibilité d'utiliser les techniques classiques de quantifications des marqueurs de différenciation cellulaire. Au niveau génétique, nous avons essayé d'extraire l'ARN a différents temps afin d'évaluer l'expression des gènes d'intérêt dans la différentiation ostéoblastique. Cependant, l'extraction d'ARN n'était pas possible à cause de la présence du GNF, comme contaminant, dans l'extrait d'ARN total. Notre hypothèse est que la présence au sein du GNF d'un groupement thymidine empêche une purification efficace des acides nucléiques par les techniques habituelles. L'absorbance du GNF a aussi été mesurée aux deux longueurs d'onde d'intérêts pour la quantification d'ARN (260 et 280 nm). On a alors observé un taux d'absorption très élevé ce qui empêche une quantification correcte d'ARN total purifié et rend donc impossible la réalisation de l'évaluation par PCR.

Au niveau protéique, nous avons essayé de réaliser des extractions de protéines afin d'évaluer par exemple l'expression de la phosphatase alcaline de façon quantitative. Plusieurs méthodes ont été utilisées (mécanique, thermique, physique) mais nous n'avons pas été en mesure d'obtenir suffisamment de protéines afin d'atteindre le seuil de détection du test.

7.2. Le développement et l'étude de nouvelles molécules gélatrices

En parallèle à nos travaux, l'équipe de chimistes à l'origine de la molécule de GNF travaillait sur le développement et les synthèses de nouvelles molécules gélatrices comme le GNBA, de la même famille que le GNF[381]. Nous avons participé à la caractérisation biologique de ces nouveaux composés et les premiers résultats sont présentés sous forme d'une publication présentée en annexe[381]. L'hydrogel formé par cette nouvelle molécule n'est pas cytotoxique. De plus il permet l'adhésion et la survie des cellules ensemencées au sein du matériau pendant plusieurs jours ce qui en fait un candidat pertinent pour une application en ingénierie tissulaire. Les résultats obtenus suite à la réalisation de ces travaux sont présentés en annexe de ce manuscrit.

7.3. Les cellules souches issues du tissu adipeux

Lors de certaines chirurgies réalisées au CHU de Bordeaux, nous récupérions le tissu adipeux prélevé et habituellement destiné aux déchets opératoires (approbation éthique DC-2008-412). Nous obtenions à partir de ces tissus des cellules souches issues du tissu adipeux (hASCs); ce sont des cellules primaires. Par définition celles-ci proviennent directement d'un organisme et ne subissent pas de modifications permettant par exemple de les rendre immortelles comme c'est le cas pour les cellules dites de

« lignées ». L'intérêt majeur de l'utilisation de ces cellules est qu'elle permet, dans une optique à long terme, de transférer les stratégies développées à une application clinique éventuelle : utiliser les cellules de façon autologue et ainsi limiter les risques liés à l'immuno-incompatibilité. Cependant nous avons été confrontés à certaines limites liées à l'utilisation de cellules primaires. En effet ces cellules sont disponibles au rythme des interventions chirurgicales et leur fragilité exige une attention particulière lors de leur manipulation. Pendant les travaux de thèses nous avons eu une importante contamination de nos stocks d'hASCs par des mycoplasmes. Ceci a considérablement impacté sur les résultats obtenus *in vitro* et *in vivo*. Tout comme pour les contaminations des poudres nécessaires à la fabrication des hydrogels nous avons tiré des enseignements : désormais chaque lot de cellules est testé par PCR pour s'assurer de la non-contamination des cellules avec lesquelles nous travaillons. C'est d'ailleurs lors de la période de décontamination du laboratoire et des stocks cellulaires qu'il a été proposé de s'intéresser aux capacités de l'hydrogel à délivrer un facteur de croissance, cette stratégie nous permettant de nous affranchir de l'utilisation de cellules.

7.4. Les modèles d'étude *in vivo*

Comme décrit précédemment nous avons étudié les propriétés de nos matériaux *in vivo* par deux modèles chez la souris : en site sous cutané (ectopique) et avec la réalisation de lésion de la calvaria (orthotopique). Bien que ces techniques aient permis de mettre en évidence les propriétés ostéoinductives et ostéoconductives des matériaux développés, elles présentent des limites. Tout d'abord d'un point de vue anatomique la calvaria est un os plat, de structure particulière, puisqu'il ne contient pas de corticale (ou cortex) ni de travées osseuses. Ensuite une limite de la lésion de calvaria réside dans l'absence quasi-totale de mises en charge. Or nous savons que les contraintes mécaniques jouent un rôle primordial dans les phases de cicatrisation et de remodelage osseux[450]. Il serait alors pertinent d'étudier les capacités de régénération osseuse dans des modèles de résections osseuses sur des fémurs de rat par exemple. Dans ce type d'étude il serait alors nécessaire de stabiliser la fracture puis de maintenir l'hydrogel avec une membrane afin que celui-ci reste en place au sein de la lésion. Pour suivre le processus de régénération osseuse de façon longitudinale et avoir une référence correspondant à l'état initial de la lésion[450]. Il est nécessaire dans ce type d'étude de disposer d'un micro-scanner sur site, ce qui n'était pas le cas au moment où nous avons réalisé nos travaux. Ce type d'appareillage aurait également permis de réaliser des images à différents intervalles dans le cas des implantations sous cutanées afin d'ajouter une dimension temporelle à notre étude. Il aurait été ainsi possible de savoir quand

apparaissent les premières étapes de minéralisation du gel et quand cette minéralisation atteignait son maximum.

8. Conclusion

Ce travail de doctorat s'est déroulé entre deux laboratoires, dans un contexte pluridisciplinaire puisque les travaux menés ont requis des notions de biologie, de chimie, de sciences des matériaux et du vivant. Les recherches se sont focalisées sur la mise au point, l'étude et la validation *in vitro* puis *in vivo* de stratégies pour l'ingénierie tissulaire osseuse.

Au cours de ces travaux, plusieurs difficultés ont été rencontrées et des stratégies ont été étudiées et mises en place pour les surmonter. Ces difficultés étaient liées à l'utilisation de matériaux dont les méthodes de fabrication et de conservations n'étaient pas encore totalement établies, conduisant à une évolution de leurs propriétés soit d'un lot à l'autre, soit au cours du temps pour un même lot. Cela a nécessité des étapes de re-purification. Malgré ces limites, nos travaux ont permis de proposer des stratégies pour la régénération osseuse basées sur l'utilisation d'hydrogels de GNF. Nos travaux ont mis en évidence les propriétés rhéologiques du gel de GNF pour une injection et une manipulation aisée du produit, permettant d'y incorporer une matrice naturelle contenant des cellules ou des molécules bioactives. La formation du gel est obtenue en refroidissant naturellement la solution de GNF, évitant toute réticulation chimique qui nécessiterait des réactifs potentiellement toxiques. De plus, la gélification se produit lentement, permettant un l'ajout des cellules ou des molécules bioactives à 37 ° C avant que la formation du gel ne soit terminée. Cette propriété offre également la possibilité d'injecter la solution de GNF encore visqueuse et de la laisser gélifier *in situ*. Compte tenu des propriétés du gel de GNF qui ont été décrites et étudiées, ce composé apparaît comme un biomatériau prometteur pour des stratégies d'ITO qui reposent sur l'apport de cellules ou de molécules bioactives à la lésion osseuse. D'autres perspectives sont l'utilisation de ce gel pour différentes applications d'ingénierie tissulaire. En effet, il serait possible d'associer l'hydrogel de GNF à d'autres matrices naturelles ou molécules bioactives en vue d'étudier ses capacités à régénérer d'autres types de tissus lésés.

Bibliographie

[1] S. Standring, Gray's Anatomy, 41st Edition, Elsevier, New York, 2015.

[2] S.P. Samuel, G.R. Baran, Y. Wei, B.L. Davis, Biomechanics - Part II, in: J.S. Khurana (Ed.), Bone Pathol., Humana Press, Totowa, NJ, 2009: pp. 69–77. doi:10.1007/978-1-59745-347-9_4.

[3] E.N. Marieb, Essentials of human anatomy and physiology, 2009.

[4] S.C. Cowin, Bone poroelasticity, J. Biomech. 32 (1999) 217–238. doi:10.1016/S0021-9290(98)00161-4.

[5] Currey JD., Bones: structure and mechanics, 2002. doi:10.1016/S0021-9290(03)00033-2.

[6] S. Viguet-Carrin, P. Garnero, P.D. Delmas, The role of collagen in bone strength, Osteoporos. Int. 17 (2006) 319–336. doi:10.1007/s00198-005-2035-9.

[7] X. Liu, H. Wu, M. Byrne, S. Krane, R. Jaenisch, Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development., Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1852–6. doi:10.1073/pnas.94.5.1852.

[8] R.J. Wenstrup, J.B. Florer, E.W. Brunskill, S.M. Bell, I. Chervoneva, D.E. Birk, Type V collagen controls the initiation of collagen fibril assembly, J. Biol. Chem. 279 (2004) 53331–53337. doi:10.1074/jbc.M409622200.

[9] Chapter 6: Osseous Tissue and Bone Structure Flashcards | Easy Notecards, (n.d.). http://www.easynotecards.com/notecard_set/18419.

[10] S. Mathews, S.A. Mathew, P.K. Gupta, R. Bhonde, S. Totey, Glycosaminoglycans enhance osteoblast differentiation of bone marrow derived human mesenchymal stem cells, J. Tissue Eng. Regen. Med. 8 (2014) 143–152. doi:10.1002/term.1507.

[11] F. Lamoureux, M. Baud'huin, L. Duplomb, D. Heymann, F. Redini, Proteoglycans: Key partners in bone cell biology, BioEssays. 29 (2007) 758–771. doi:10.1002/bies.20612.

[12] R. Tamma, C. Carbone, S. Colucci, Bone matrix proteins and mineralization process, in: C. V Albanese, C. Faletti (Eds.), Imaging Prosthet. Joints A Comb. Radiol. Clin. Perspect., Springer Milan, Milano, 2014: pp. 15–25. doi:10.1007/978-88-470-5483-7_2.

[13] Y. Imai, M.-Y. Youn, K. Inoue, I. Takada, A. Kouzmenko, S. Kato, Nuclear Receptors in Bone Physiology and Diseases, Physiol. Rev. 93 (2013).

[14] L. Polo-Corrales, M. Latorre-Esteves, J.E. Ramirez-Vick, Scaffold design for bone regeneration., J. Nanosci. Nanotechnol. 14 (2014) 15–56. doi:10.1166/jnn.2014.9127.

[15] S.Y. Tang, T. Alliston, Regulation of postnatal bone homeostasis by TGFβ, Bonekey Rep. 2 (2013) 255. doi:10.1038/bonekey.2012.255.

[16] L.F. Bonewald, The Role of the Osteocyte in Bone and Nonbone Disease, Endocrinol. Metab. Clin. North Am. 46 (2017) 1–18. doi:10.1016/j.ecl.2016.09.003.

[17] S.C. Miller, L. de Saint-Georges, B.M. Bowman, W.S. Jee, Bone lining cells: structure and function., Scanning Microsc. 3 (1989) 953–60.

[18] B. Clarke, Normal bone anatomy and physiology., Clin. J. Am. Soc. Nephrol. 3 Suppl 3 (2008) S131–S139. doi:10.2215/CJN.04151206.

[19] S.L. Teitelbaum, F.P. Ross, Genetic regulation of osteoclast development and function., Nat. Rev. Genet. 4 (2003) 638–49. doi:10.1038/nrg1122.

[20] F. Lioté, C. Nguyen, T. Funck-Brentano, M. Cohen-Solal, L'aube des biothérapies de l'ostéoporose et leur intérêt dans les rhumatismes inflammatoires, in: Biothèrapies En Rhumatol., Springer Paris, Paris, 2011: pp. 131–145. doi:10.1007/978-2-8178-0124-7_10.

[21] C. Carulli, M. Innocenti, M.L. Brandi, Bone vascularization in normal and disease conditions, Front. Endocrinol. (Lausanne). 4 (2013) 106. doi:10.3389/fendo.2013.00106.

[22] M. Laroche, Intraosseous circulation from physiology to disease, Jt. Bone Spine. 69 (2002) 262–269. doi:10.1016/S1297-319X(02)00391-3.

[23] K.K. Hirschi, P.A. D'Amore, Pericytes in the microvasculature, Cardiovasc. Res. 32 (1996) 687–698. doi:10.1016/0008-6363(96)00063-6.

[24] F. Villars, B. Guillotin, T. Amédée, S. Dutoya, L. Bordenave, R. Bareille, J. Amédée, Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication., Am. J. Physiol. Cell Physiol. 282 (2002) C775-85. doi:10.1152/ajpcell.00310.2001.

[25] Y. Wallez, I. Vilgrain, P. Huber, Angiogenesis: The VE-cadherin switch, Trends Cardiovasc. Med. 16 (2006) 55–59. doi:10.1016/j.tcm.2005.11.008.

[26] M.C. Siebers, P.J. Ter Brugge, X.F. Walboomers, J.A. Jansen, Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review, Biomaterials. 26 (2005) 137–146. doi:10.1016/j.biomaterials.2004.02.021.

[27] AboutKidsHealth, about.kidshealth@sickkids.ca, Blood Circulation to the Bones, (n.d.). http://www.aboutkidshealth.ca/BCCH/En/HowTheBodyWorks/IntroductiontotheSkeleton/BloodCirculationtotheBones/Pages/default.aspx (accessed April 29, 2017).

[28] J.-M. Delaisse, The reversal phase of the bone-remodeling cycle: cellular prerequisites for coupling resorption and formation., Bonekey Rep. 3 (2014) 561. doi:10.1038/bonekey.2014.56.

[29] L.J. Raggatt, N.C. Partridge, Cellular and molecular mechanisms of bone remodeling, J. Biol. Chem. 285 (2010) 25103–25108. doi:10.1074/jbc.R109.041087.

[30] X. Feng, J.M. McDonald, Disorders of bone remodeling., in: A.K. Abbas, S.J. Galli, P.M. Howley (Eds.), Annu. Rev. Pathol., Annual Reviews, Palo Alto, 2011: pp. 121–45. doi:10.1146/annurev-pathol-011110-130203.

[31] K.N. Weilbaecher, T.A. Guise, L.K. McCauley, Cancer to bone: a fatal attraction, Nat. Rev. Cancer. 11 (2011) 411–425. doi:10.1038/nrc3055.

[32] N. Baldini, E. Cenni, G. Ciapetti, D. Granchi, L. Savarino, Bone repair and regeneration, Bone Repair Biomater. (2009) 69–105. doi:10.1533/9781845696610.1.69.

[33] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, Nat. Rev. Rheumatol. 11 (2014) 45–54. doi:10.1038/nrrheum.2014.164.

[34] L. Marzona, B. Pavolini, Play and players in bone fracture healing match, Clin. Cases Miner. Bone Metab. 6 (2009) 159–162.

[35] R. Marsell, T.A. Einhorn, The biology of fracture healing, Injury. 42 (2011) 551–555. doi:10.1016/j.injury.2011.03.031.

[36] G.O. Matheson, D.B. Clement, D.C. McKenzie, J.E. Taunton, D.R. Lloyd-Smith, J.G. MacIntyre, Stress fractures in athletes. A study of 320 cases., Am. J. Sports Med. 15 (1971) 46–58. doi:10.1177/036354658701500107.

[37] J.M. Maes, G. Raoul, M. Omezzine, J. Ferri, Ostéites des os de la face, EMC-Stomatologie. 1 (2005) 208–230. doi:10.1016/j.emcsto.2005.07.004.

[38] C. Proust, J. Proust, A. Maubon, Imagerie des métastases osseuses, Médecine Nucléaire - Imag. Fonct. Métabolique. 30 (2006) 30–3.

[39] F. Chotel, Résection chirurgicale ou « carcinologique » des tumeurs osseuses malignes, 2010.

[40] P. Page, Osteoporosis Handout on Health, Search. (2010) 1–15. https://www.niams.nih.gov/health_info/Osteoporosis/default.asp.

[41] A.L. Golob, M.B. Laya, Osteoporosis: Screening, Prevention, and Management, Med. Clin. North Am. 99 (2015) 587–606. doi:10.1016/j.mcna.2015.01.010.

[42] M. Nevins, J.T. Mellonig, The advantages of localized ridge augmentation prior to implant placement: a staged event., Int. J. Periodontics Restorative Dent. 14 (1994) 96–111.

[43] P. Bravetti, Les comblements osseux et alternatives aux comblements, LS - La Lett. La Stomatol. (2015) 1–20.

[44] A. Kinaci, V. Neuhaus, D.C. Ring, Trends in bone graft use in the United States., Orthopedics. 37 (2014) e783-8. doi:10.3928/01477447-20140825-54.

[45] J.W. Canady, D.P. Zeitler, S.A. Thompson, C.D. Nicholas, Suitability of the iliac crest as a site for harvest of autogenous bone grafts, Cleft Palate-Craniofacial J. 30 (1993) 579–581. doi:10.1597/1545-1569(1993)030<0579:SOTICA>2.3.CO;2.

[46] P.N. Soucacos, A. V. Korompilias, M.D. Vekris, A. Zoubos, A.E. Beris, The free vascularized fibular graft for bridging large skeletal defects of the upper extremity, Microsurgery. 31 (2011) 190–197. doi:10.1002/micr.20862.

[47] H.T. Aro, a J. Aho, Clinical use of bone allografts., Ann. Med. 25 (1993) 403–412. doi:10.3109/07853899309147303.

[48] T. Eastlund, Infectious disease transmission through cell, tissue, and organ transplantation: Reducing the risk through donor selection, Cell Transplant. 4 (1995) 455–477. doi:10.1016/0963-6897(95)00035-V.

[49] P. Kumar, B. Vinitha, G. Fathima, Bone grafts in dentistry., J. Pharm. Bioallied Sci. 5 (2013) S125-7. doi:10.4103/0975-7406.113312.

[50] R.R. Pelker, G.E. Friedlaender, T.C. Markham, Biomechanical properties of bone allografts., Clin. Orthop. Relat. Res. (1983) 54–7.

[51] D. Chappard, A. Zhioua, F. Grizon, M.F. Basle, A. Rebel, [Biomaterials for bone filling: comparisons between autograft, hydroxyapatite and one highly purified bovine xenograft], Bull.Assoc.Anat.(Nancy.). 77 (1993) 59–65.

[52] S. Stevenson, S.E. Emery, V.M. Goldberg, Factors affecting bone graft incorporation., Clin.

Orthop. Relat. Res. (1996) 66–74.

[53] S.P. Cowley, L.D. Anderson, Hernias through donor sites for iliac-bone grafts., J. Bone Joint Surg. Am. 65 (1983) 1023–5.

[54] K.L. Brown, R.L. Cruess, Bone and cartilage transplantation in orthopaedic surgery. A review., J. Bone Joint Surg. Am. 64 (1982) 270–9.

[55] C.J. Damien, J.R. Parsons, Bone graft and bone graft substitutes: A review of current technology and applications, J. Appl. Biomater. 2 (1991) 187–208. doi:10.1002/jab.770020307.

[56] E.M. Younger, M.W. Chapman, Morbidity at bone graft donor sites., J. Orthop. Trauma. 3 (1989) 192–5.

[57] A.G. Coombes, M.C. Meikle, Resorbable synthetic polymers as replacements for bone graft., Clin. Mater. 17 (1994) 35–67.

[58] J.M. Lane, E. Tomin, M.P. Bostrom, Biosynthetic bone grafting., Clin. Orthop. Relat. Res. (1999) S107-17.

[59] M.R. Urist, B.F. Silverman, K. Büring, F.L. Dubuc, J.M. Rosenberg, The bone induction principle., Clin. Orthop. Relat. Res. 53 (n.d.) 243–83.

[60] M.L. Anderson, W.J. Dhert, J.D. de Bruijn, R.A. Dalmeijer, H. Leenders, C.A. van Blitterswijk, A.J. Verbout, Critical size defect in the goat's os ilium. A model to evaluate bone grafts and substitutes., Clin. Orthop. Relat. Res. (1999) 231–9.

[61] H. Sciences, Craniofacial Tissue Engineering, Crit. Rev. Oral Biol. Med. 2 (2015) 64–75. doi:10.1177/10454411010120010501.

[62] H. Green, O. Kehinde, J. Thomas, Growth of cultured human epidermal cells into multiple epithelia suitable for grafting., Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 5665–8. doi:10.1073/pnas.76.11.5665.

[63] G.G. Gallico, N.E. O'Connor, C.C. Compton, O. Kehinde, H. Green, Permanent coverage of large burn wounds with autologous cultured human epithelium, N. Engl. J. Med. 311 (1984) 448–451. doi:10.1056/NEJM198408163110706.

[64] J. Tien, C.M. Nelson, Microstructured extracellular matrices in tissue engineering and development: An update, Ann. Biomed. Eng. 42 (2014) 1413–1423. doi:10.1007/s10439-013-0912-5.

[65] K.E. Healy, R.E. Guldberg, Bone tissue engineering., J. Musculoskelet. Neuronal Interact. 7 (2007) 328–330. doi:http://dx.doi.org/10.1016/j.oooo.2012.02.030.

[66] J. Zhou, H. Lin, T. Fang, X. Li, W. Dai, T. Uemura, J. Dong, The repair of large segmental bone defects in the rabbit with vascularized tissue engineered bone, Biomaterials. 31 (2010) 1171–1179. doi:10.1016/j.biomaterials.2009.10.043.

[67] G. Sun, D. Wei, X. Liu, Y. Chen, M. Li, D. He, J. Zhong, Novel biodegradable electrospun nanofibrous P(DLLA-CL) balloons for the treatment of vertebral compression fractures, Nanomedicine Nanotechnology, Biol. Med. 9 (2013) 829–838. doi:10.1016/j.nano.2012.12.003.

[68] J. Silva-Correia, S.I. Correia, J.M. Oliveira, R.L. Reis, Tissue engineering strategies applied in the regeneration of the human intervertebral disk, Biotechnol. Adv. 31 (2013) 1514–1531. doi:10.1016/j.biotechadv.2013.07.010.

[69] N. Pallua, C. V. Suschek, Tissue engineering: From lab to clinic, Tissue Eng. From Lab to Clin. 260 (2011) 1–634. doi:10.1007/978-3-642-02824-3.

[70] A.B. Hart, D.C. Samuels, T. Hulgan, The other genome: A systematic review of studies of mitochondrial DNA haplogroups and outcomes of HIV infection and antiretroviral therapy, AIDS Rev. 15 (2013) 213–220. doi:10.1037/a0030561.Striving.

[71] R. Tevlin, G.G. Walmsley, O. Marecic, M.S. Hu, D.C. Wan, M.T. Longaker, Stem and progenitor cells: advancing bone tissue engineering, Drug Deliv. Transl. Res. 6 (2016) 159–173. doi:10.1007/s13346-015-0235-1.

[72] J. Ma, S.K. Both, F. Yang, F.-Z. Cui, J. Pan, G.J. Meijer, J. a Jansen, J.J.J.P. van den Beucken, Concise review: cell-based strategies in bone tissue engineering and regenerative medicine., Stem Cells Transl. Med. 3 (2014) 98–107. doi:10.5966/sctm.2013-0126.

[73] K. Lee, E.A. Silva, D.J. Mooney, Growth factor delivery-based tissue engineering: general approaches and a review of recent developments., J. R. Soc. Interface. 8 (2011) 153–70. doi:10.1098/rsif.2010.0223.

[74] L.J. Bonassar, C. a Vacanti, Tissue engineering: the first decade and beyond., J. Cell. Biochem. Suppl. 30–31 (1998) 297–303. doi:10.1002/(SICI)1097-4644(1998)72.

[75] O. Schultz, M. Sittinger, T. Haeupl, G.R. Burmester, Emerging strategies of bone and joint repair., Arthritis Res. 2 (2000) 433–436. doi:10.1186/ar123.

[76] M. Swetha, K. Sahithi, A. Moorthi, N. Srinivasan, K. Ramasamy, N. Selvamurugan, Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering, Int. J. Biol. Macromol. 47 (2010) 1–4. doi:10.1016/j.ijbiomac.2010.03.015.

[77] N. Datta, Q.P. Pham, U. Sharma, V.I. Sikavitsas, J.A. Jansen, A.G. Mikos, In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2488–93. doi:10.1073/pnas.0505661103.

[78] K.Y. Lee, E. Alsberg, D.J. Mooney, Degradable and injectable poly(aldehyde guluronate) hydrogels for bone tissue engineering, J. Biomed. Mater. Res. 56 (2001) 228–233. doi:10.1002/1097-4636(200108)56:2<228::AID-JBM1089>3.0.CO;2-9.

[79] H. Brøndsted, J. Kopeček, Hydrogels for Site-Specific Drug Delivery to the Colon: In Vitro and in Vivo Degradation, Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci. 9 (1992) 1540–1545. doi:10.1023/A:1015847921435.

[80] K.H. Bouhadir, G.M. Kruger, K.Y. Lee, D.J. Mooney, Sustained and controlled release of daunomycin from cross-linked poly(aldehyde guluronate) hydrogels, J. Pharm. Sci. 89 (2000) 910–919. doi:10.1002/1520-6017(200007)89:7<910::AID-JPS8>3.0.CO;2-#.

[81] M.S. Jhon, J.D. Andrade, Water and hydrogels, J. Biomed. Mater. Res. 7 (1973) 509–522. doi:10.1002/jbm.820070604.

[82] A.C. Jen, M.C. Wake, A.G. Mikos, Review: Hydrogels for cell immobilization, Biotechnol. Bioeng. 50 (1996) 357–364. doi:10.1002/(SICI)1097-0290(19960520)50:4<357::AID-BIT2>3.0.CO;2-K.

[83] J.A. Rowley, G. Madlambayan, D.J. Mooney, Alginate hydrogels as synthetic extracellular matrix materials, Biomaterials. 20 (1999) 45–53. doi:10.1016/S0142-9612(98)00107-0.

[84] J. Park, R.S. Lakes, Biomaterials: An introduction: Third edition, 2007. doi:10.1007-978-0-387-37880-0.

[85] J.M. Anderson, Chapter 4 Mechanisms of inflammation and infection with implanted devices, Cardiovasc. Pathol. 2 (1993) 33–41. doi:10.1016/1054-8807(93)90045-4.

[86] D.F. Williams, A model for biocompatibility and its evaluation, J. Biomed. Eng. 11 (1989) 185–191. doi:10.1016/0141-5425(89)90138-6.

[87] H. Zigdon-Giladi, U. Rudich, G. Michaeli Geller, A. Evron, Recent advances in bone regeneration using adult stem cells., World J. Stem Cells. 7 (2015) 630–40. doi:10.4252/wjsc.v7.i3.630.

[88] V.M.C. Quent, C. Theodoropoulos, D.W. Hutmacher, J.C. Reichert, Differential osteogenicity of multiple donor-derived human mesenchymal stem cells and osteoblasts in monolayer, scaffold-based 3D culture and in vivo, Biomed. Tech. 61 (2016) 253–266. doi:10.1515/bmt-2014-0159.

[89] F. Cecchinato, J. Karlsson, L. Ferroni, C. Gardin, S. Galli, A. Wennerberg, B. Zavan, M. Andersson, R. Jimbo, Osteogenic potential of human adipose-derived stromal cells on 3-dimensional mesoporous TiO2 coating with magnesium impregnation, Mater. Sci. Eng. C. 52 (2015) 225–234. doi:10.1016/j.msec.2015.03.026.

[90] E.E. Johnson, M.R. Urist, G. a Finerman, Repair of segmental defects of the tibia with cancellous bone grafts augmented with human bone morphogenetic protein. A preliminary report., Clin. Orthop. Relat. Res. (1988) 249–57.

[91] U. Ripamonti, N. Duneas, Tissue morphogenesis and regeneration by bone morphogenetic proteins., Plast. Reconstr. Surg. 101 (1998) 227–239. doi:10.1097/00006534-199801000-00040.

[92] J.M. Granjeiro, R.C. Oliveira, J.C. Bustos-Valenzuela, M.C. Sogayar, R. Taga, Bone morphogenetic proteins: From structure to clinical use, Brazilian J. Med. Biol. Res. 38 (2005) 1463–1473. doi:10.1590/S0100-879X2005001000003.

[93] S.-J. Huang, R.-H. Fu, W.-C. Shyu, S.-P. Liu, G.-P. Jong, Y.-W. Chiu, H.-S. Wu, Y.-A. Tsou, C.-W. Cheng, S.-Z. Lin, Adipose-Derived Stem Cells: Isolation, Characterization, and Differentiation Potential, Cell Transplant. 22 (2013) 701–709. doi:10.3727/096368912X655127.

[94] P. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Furtell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hendrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2004) 211–228.

[95] Y.D. Halvorsen, D. Franklin, A.L. Bond, D.C. Hitt, C. Auchter, A.L. Boskey, E.P. Paschalis, W.O. Wilkison, J.M. Gimble, Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells., Tissue Eng. 7 (2001) 729–741. doi:10.1089/107632701753337681.

[96] R. Ogawa, H. Mizuno, A. Watanabe, M. Migita, T. Shimada, H. Hyakusoku, Osteogenic and chondrogenic differentiation by adipose-derived stem cells harvested from GFP transgenic mice, Biochem. Biophys. Res. Commun. 313 (2004) 871–877. doi:10.1016/j.bbrc.2003.12.017.

[97] H.A. Awad, M.Q. Wickham, H.A. Leddy, J.M. Gimble, F. Guilak, Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds, Biomaterials. 25 (2004) 3211–3222. doi:10.1016/j.biomaterials.2003.10.045.

[98] G.R. Erickson, J.M. Gimble, D.M. Franklin, H.E. Rice, H. Awad, F. Guilak, Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo, Biochem Biophys Res Commun. 290 (2002) 763–769. doi:10.1006/bbrc.2001.6270\rS0006291X01962709 [pii].

[99] H. Mizuno, P.A. Zuk, M. Zhu, H.P. Lorenz, P. Benhaim, M.H. Hedrick, Myogenic Differentiation by Human Processed Lipoaspirate Cells, Plast. Reconstr. Surg. 109 (2002) 199–209.

doi:10.1097/00006534-200201000-00031.

[100] A.M. DiMarino, A.I. Caplan, T.L. Bonfield, Mesenchymal Stem Cells in Tissue Repair, Front. Immunol. 4 (2013) 201. doi:10.3389/fimmu.2013.00201.

[101] K.M. Safford, K.C. Hicok, S.D. Safford, Y.-D.C. Halvorsen, W.O. Wilkison, J.M. Gimble, H.E. Rice, Neurogenic differentiation of murine and human adipose-derived stromal cells, Biochem. Biophys. Res. Commun. 294 (2002) 371–379. doi:10.1016/S0006-291X(02)00469-2.

[102] M.Q. Wickham, G.R. Erickson, J.M. Gimble, T.P. Vail, F. Guilak, Multipotent stromal cells derived from the infrapatellar fat pad of the knee., Clin. Orthop. Relat. Res. (2003) 196–212. doi:10.1097/01.blo.0000072467.53786.ca.

[103] H. Liao, C.-T. Chen, Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells, World J. Stem Cells. 6 (2014) 288. doi:10.4252/wjsc.v6.i3.288.

[104] J.A. Lee, B.M. Parrett, J.A. Conejero, J. Laser, J. Chen, A.J. Kogon, D. Nanda, R.T. Grant, A.S. Breitbart, Biological alchemy: engineering bone and fat from fat-derived stem cells., Ann. Plast. Surg. 50 (2003) 610–7. doi:10.1097/01.SAP.0000069069.23266.35.

[105] M. Achilli, D. Mantovani, Tailoring mechanical properties of collagen-based scaffolds for vascular tissue engineering: The effects of pH, temperature and ionic strength on gelation, Polymers (Basel). 2 (2010) 664–680. doi:10.3390/polym2040664.

[106] M. Achilli, J. Lagueux, D. Mantovani, On the effects of UV-C and pH on the mechanical behavior, molecular conformation and cell viability of collagen-based scaffold for vascular tissue engineering, Macromol. Biosci. 10 (2010) 307–316. doi:10.1002/mabi.200900248.

[107] F. Boccafoschi, J. Habermehl, S. Vesentini, D. Mantovani, Biological performances of collagen-based scaffolds for vascular tissue engineering, Biomaterials. 26 (2005) 7410–7417. doi:10.1016/j.biomaterials.2005.05.052.

[108] J.M. MILLER, Clinical Observations, Arch. Surg. 88 (1964) 167. doi:10.1001/archsurg.1964.01310200005002.

[109] I.W. Browder, M.S. Litwin, Use of absorbable collagen for hemostasis in general surgical patients, Am. Surg. 52 (1986) 492–494.

[110] H.E. KAUFMAN, T.L. STEINEMANN, E. LEHMAN, H.W. THOMPSON, E.D. VARNELL, J.T. JACOB-LaBARRE, B.M. GEBHARDT, Collagen-Based Drug Delivery and Artificial Tears, J. Ocul. Pharmacol. Ther. 10 (1994) 17–27. doi:10.1089/jop.1994.10.17.

[111] P.A. Lucas, G.T. Syftestad, V.M. Goldberg, A.I. Caplan, Ectopic induction of cartilage and bone by water-soluble proteins from bovine bone using a collagenous delivery vehicle, J. Biomed. Mater. Res. 23 (1989) 23–39. doi:10.1002/jbm.820231306.

[112] K. Panduranga Rao, Recent developments of collagen-based materials for medical applications and drug delivery systems, J. Biomater. Sci. Polym. Ed. 7 (1996) 623–645. doi:10.1163/156856295X00526.

[113] R.J. Mullins, C. Richards, T. Walker, Allergic reactions to oral, surgical and topical bovine collagen. Anaphylactic risk for surgeons., Aust. N. Z. J. Ophthalmol. 24 (1996) 257–260. doi:10.1111/j.1442-9071.1996.tb01589.x.

[114] A.K. Lynn, I. V. Yannas, W. Bonfield, Antigenicity and immunogenicity of collagen, J. Biomed. Mater. Res. - Part B Appl. Biomater. 71 (2004) 343–354. doi:10.1002/jbm.b.30096.

[115] N. Rajan, J. Habermehl, M.-F. Coté, C.J. Doillon, D. Mantovani, Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications., Nat. Protoc. 1 (2006) 2753–2758. doi:10.1038/nprot.2006.430.

[116] S.D. McCullen, P.R. Miller, S.D. Gittard, R.E. Gorga, B. Pourdeyhimi, R.J. Narayan, E.G. Loboa, *In Situ* Collagen Polymerization of Layered Cell-Seeded Electrospun Scaffolds for Bone Tissue Engineering Applications, Tissue Eng. Part C Methods. 16 (2010) 1095–1105. doi:10.1089/ten.tec.2009.0753.

[117] Y. Isobe, T. Kosaka, G. Kuwahara, H. Mikami, T. Saku, S. Kodama, Oriented Collagen Scaffolds for Tissue Engineering, Materials (Basel). 5 (2012) 501–511. doi:10.3390/ma5030501.

[118] W. He, T. Yong, W.E. Teo, Z. Ma, S. Ramakrishna, Fabrication and endothelialization of collagen-blended biodegradable polymer nanofibers: potential vascular graft for blood vessel tissue engineering., Tissue Eng. 11 (2005) 1574–1588. doi:10.1089/ten.2005.11.1574.

[119] M.M. Stevens, Biomaterials for bone tissue engineering, Mater. Today. 11 (2008) 18–25. doi:10.1016/S1369-7021(08)70086-5.

[120] G. Godeau, J. Bernard, C. Staedel, P. Barthélémy, Glycosyl-nucleoside-lipid based supramolecular assembly as a nanostructured material with nucleic acid delivery capabilities., Chem. Commun. (Camb). (2009) 5127–5129. doi:10.1039/b906212b.

[121] G. Godeau, C. Brun, H. Arnion, C. Staedel, P. Barthélémy, Glycosyl-nucleoside fluorinated amphiphiles as components of nanostructured hydrogels, 2010. doi:10.1016/j.tetlet.2009.12.042.

[122] C. Vinatier, J. Guicheux, G. Daculsi, P. Layrolle, P. Weiss, Cartilage and bone tissue engineering using hydrogels., Biomed Mater Eng. 16 (2006) S107-13.

[123] S. Ziane, S. Schlaubitz, S. Miraux, A. Patwa, C. Lalande, I. Bilem, S. Lepreux, B. Rousseau, J.F. Le Meins, L. Latxague, P. Barthélémy, O. Chassande, A thermosensitive low molecular weight hydrogel as scaffold for tissue engineering, Eur. Cells Mater. 23 (2012) 147–160.

[124] A.B.J. Prowse, F. Chong, P.P. Gray, T.P. Munro, Stem cell integrins: Implications for ex-vivo culture and cellular therapies, Stem Cell Res. 6 (2011) 1–12. doi:10.1016/j.scr.2010.09.005.

[125] A. Wall, T. Board, Bone: Formation by autoinduction, Class. Pap. Orthop. 150 (2014) 449–451. doi:10.1007/978-1-4471-5451-8_114.

[126] W.F. McKay, S.M. Peckham, J.M. Badura, A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft), Int. Orthop. 31 (2007) 729–734. doi:10.1007/s00264-007-0418-6.

[127] T. Liu, Y. Gao, K. Sakamoto, T. Minamizato, K. Furukawa, T. Tsukazaki, Y. Shibata, K. Bessho, T. Komori, A. Yamaguchi, BMP-2 promotes differentiation of osteoblasts and chondroblasts in Runx2-deficient cell lines, J. Cell. Physiol. 211 (2007) 728–735. doi:10.1002/jcp.20988.

[128] M. Maisani, D. Pezzoli, O. Chassande, D. Mantovani, Cellularizing hydrogel-based scaffolds to repair bone tissue: How to create a physiologically relevant micro-environment?, J. Tissue Eng. 8 (2017) 204173141771207. doi:10.1177/2041731417712073.

[129] J. Stanovici, L.R. Le Nail, M.A. Brennan, L. Vidal, V. Trichet, P. Rosset, P. Layrolle, Bone regeneration strategies with bone marrow stromal cells in orthopaedic surgery, Curr Res Transl Med. 64 (2016) 83–90. doi:10.1016/j.retram.2016.04.006.

[130] K.E. Zakhary, J.S. Thakker, Emerging Biomaterials in Trauma, Oral Maxillofac. Surg. Clin. North

Am. 29 (2017) 51–62. doi:10.1016/j.coms.2016.08.010.

[131] P. Shakoori, Q. Zhang, A.D. Le, Applications of Mesenchymal Stem Cells in Oral and Craniofacial Regeneration, Oral Maxillofac. Surg. Clin. North Am. 29 (2017) 19–25. doi:10.1016/j.coms.2016.08.009.

[132] D. Rolski, J. Kostrzewa-Janicka, P. Zawadzki, K. Życińska, E. Mierzwińska-Nastalska, The Management of Patients after Surgical Treatment of Maxillofacial Tumors., Biomed Res. Int. 2016 (2016) 4045329. doi:10.1155/2016/4045329.

[133] L. Larsson, A.M. Decker, L. Nibali, S.P. Pilipchuk, T. Berglundh, W. V Giannobile, Regenerative Medicine for Periodontal and Peri-implant Diseases., J. Dent. Res. 95 (2016) 255–66. doi:10.1177/0022034515618887.

[134] A. Sivashanmugam, R. Arun Kumar, M. Vishnu Priya, S. V. Nair, R. Jayakumar, An overview of injectable polymeric hydrogels for tissue engineering, Eur. Polym. J. 72 (2015) 543–565. doi:10.1016/j.eurpolymj.2015.05.014.

[135] G. Vadalà, F. Russo, L. Ambrosio, M. Loppini, V. Denaro, Stem cells sources for intervertebral disc regeneration., World J. Stem Cells. 8 (2016) 185–201. doi:10.4252/wjsc.v8.i5.185.

[136] G. Tozzi, A. De Mori, A. Oliveira, M. Roldo, Composite hydrogels for bone regeneration, Materials (Basel). 9 (2016) 267. doi:10.3390/ma9040267.

[137] R. Yunus Basha, S.K. Sampath, M. Doble, Design of biocomposite materials for bone tissue regeneration, Mater. Sci. Eng. C. 57 (2015) 452–463. doi:10.1016/j.msec.2015.07.016.

[138] O.B. Betz, V.M. Betz, A. Abdulazim, R. Penzkofer, B. Schmitt, C. Schroder, S. Mayer-Wagner, P. Augat, V. Jansson, P.E. Muller, The Repair of Critical Size Bone Defects using Expedited, Autologous BMP-2 Gene Activated Fat Implants, Tissue Eng Part A. 16 (2009) 1093–1101. doi:10.1089/ten.TEA.2009.0204.

[139] A.R. Amini, C.T. Laurencin, S.P. Nukavarapu, Bone tissue engineering: recent advances and challenges., Crit. Rev. Biomed. Eng. 40 (2012) 363–408. doi:10.1615/CritRevBiomedEng.v40.i5.10.

[140] X. Wang, Y. Wang, W. Gou, Q. Lu, J. Peng, S. Lu, Role of mesenchymal stem cells in bone regeneration and fracture repair: A review, Int. Orthop. 37 (2013) 2491–2498. doi:10.1007/s00264-013-2059-2.

[141] N. Sasaki, N. Matsushima, T. Ikawa, H. Yamamura, A. Fukuda, Orientation of bone mineral and its role in the anisotropic mechanical properties of bone-Transverse anisotropy, J. Biomech. 22 (1989) 157-. doi:10.1016/0021-9290(89)90038-9.

[142] M.N. Knight, K.D. Hankenson, Mesenchymal Stem Cells in Bone Regeneration., Adv. Wound Care. 2 (2013) 306–316. doi:10.1089/wound.2012.0420.

[143] F. Granero-Moltó, J.A. Weis, M.I. Miga, B. Landis, T.J. Myers, L. O'Rear, L. Longobardi, E.D. Jansen, D.P. Mortlock, A. Spagnoli, Regenerative effects of transplanted mesenchymal stem cells in fracture healing, Stem Cells. 27 (2009) 1887–1898. doi:10.1002/stem.103.

[144] C. Yellowley, CXCL12/CXCR4 signaling and other recruitment and homing pathways in fracture repair., Bonekey Rep. 2 (2013) 300. doi:10.1038/bonekey.2013.34.

[145] I.U. Schraufstatter, R.G. Discipio, S. Khaldoyanidi, Mesenchymal stem cells and their microenvironment., Front. Biosci. (Landmark Ed. 16 (2011) 2271–88.

[146] E. Gómez-Barrena, P. Rosset, D. Lozano, J. Stanovici, C. Ermthaller, F. Gerbhard, Bone fracture healing: Cell therapy in delayed unions and nonunions, Bone. 70 (2015) 93–101. doi:10.1016/j.bone.2014.07.033.

[147] S. Sundelacruz, D.L. Kaplan, Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine, Semin. Cell Dev. Biol. 20 (2009) 646–655. doi:10.1016/j.semcdb.2009.03.017.

[148] D.F. Williams, On the mechanisms of biocompatibility, Biomaterials. 29 (2008) 2941–2953. doi:10.1016/j.biomaterials.2008.04.023.

[149] S. Bose, M. Roy, A. Bandyopadhyay, Recent advances in bone tissue engineering scaffolds, Trends Biotechnol. 30 (2012) 546–554. doi:10.1016/j.tibtech.2012.07.005.

[150] T. Albrektsson, C. Johansson, Osteoinduction, osteoconduction and osseointegration, Eur. Spine J. 10 (2001) S96–S101. doi:10.1007/s005860100282.

[151] P. Habibovic, K. de Groot, Osteoinductive biomaterials - properties and relevance in bone repair, J. Tissue Eng. Regen. Med. 1 (2007) 25–32. doi:10.1002/term.5.

[152] E. Garcia-Gareta, M.J. Coathup, G.W. Blunn, Osteoinduction of bone grafting materials for bone repair and regeneration, Bone. 81 (2015) 112–121. doi:10.1016/j.bone.2015.07.007.

[153] E. a Bayer, R. Gottardi, M. V Fedorchak, S.R. Little, The scope and sequence of growth factor delivery for vascularized bone tissue regeneration., J. Control. Release. 219 (2015) 129–40. doi:10.1016/j.jconrel.2015.08.004.

[154] H. Geckil, F. Xu, X. Zhang, S. Moon, U. Demirci, Engineering hydrogels as extracellular matrix mimics., Nanomedicine (Lond). 5 (2010) 469–84. doi:10.2217/nnm.10.12.

[155] P.J. Kondiah, Y.E. Choonara, P.P.D. Kondiah, T. Marimuthu, P. Kumar, L.C. Du Toit, V. Pillay, A review of injectable polymeric hydrogel systems for application in bone tissue engineering, Molecules. 21 (2016) 1580. doi:10.3390/molecules21111580.

[156] L.M. Marquardt, S.C. Heilshorn, Design of Injectable Materials to Improve Stem Cell Transplantation, Curr. Stem Cell Reports. 2 (2016) 1–14. doi:10.1007/s40778-016-0058-0.

[157] L. Ghasemi-Mobarakeh, Structural properties of scaffolds: Crucial parameters towards stem cells differentiation, World J. Stem Cells. 7 (2015) 728. doi:10.4252/wjsc.v7.i4.728.

[158] I. El-Sherbiny, M. Yacoub, Hydrogel scaffolds for tissue engineering: Progress and challenges, Glob. Cardiol. Sci. Pract. 2013 (2013) 316–42. doi:10.5339/gcsp.2013.38.

[159] E.M. Ahmed, Hydrogel: Preparation, characterization, and applications: A review, J. Adv. Res. 6 (2015) 105–121. doi:10.1016/j.jare.2013.07.006.

[160] L. Gasperini, J.F. Mano, R.L. Reis, Natural polymers for the microencapsulation of cells., J. R. Soc. Interface. 11 (2014) 20140817. doi:10.1098/rsif.2014.0817.

[161] K.Y. Lee, D.J. Mooney, Alginate: Properties and biomedical applications, Prog. Polym. Sci. 37 (2012) 106–126. doi:10.1016/j.progpolymsci.2011.06.003.

[162] Q. Xing, K. Yates, C. Vogt, Z. Qian, M.C. Frost, F. Zhao, Increasing mechanical strength of gelatin hydrogels by divalent metal ion removal., Sci. Rep. 4 (2014) 4706. doi:10.1038/srep04706.

[163] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, Polymer (Guildf).

49 (2008) 1993–2007. doi:10.1016/j.polymer.2008.01.027.

[164] G.J. Ritfeld, B.M. Rauck, T.L. Novosat, D. Park, P. Patel, R.A.C. Roos, Y. Wang, M. Oudega, The effect of a polyurethane-based reverse thermal gel on bone marrow stromal cell transplant survival and spinal cord repair, Biomaterials. 35 (2014) 1924–1931. doi:10.1016/j.biomaterials.2013.11.062.

[165] J.M. Orban, L.B. Wilson, J. a Kofroth, M.S. El-Kurdi, T.M. Maul, D. a Vorp, Crosslinking of collagen gels by transglutaminase., J. Biomed. Mater. Res. A. 68 (2004) 756–762. doi:10.1002/jbm.a.20110.

[166] S. Tan, J.Y. Fang, Z. Yang, M.E. Nimni, B. Han, The synergetic effect of hydrogel stiffness and growth factor on osteogenic differentiation, Biomaterials. 35 (2014) 5294–5306. doi:10.1016/j.biomaterials.2014.02.040.

[167] Y. Garcia, R. Collighan, M. Griffin, A. Pandit, Assessment of cell viability in a three-dimensional enzymatically cross-linked collagen scaffold, J. Mater. Sci. Mater. Med. 18 (2007) 1991–2001. doi:10.1007/s10856-007-3091-9.

[168] C.W. Yung, L.Q. Wu, J.A. Tullman, G.F. Payne, W.E. Bentley, T.A. Barbari, Transglutaminase crosslinked gelatin as a tissue engineering scaffold, J. Biomed. Mater. Res. - Part A. 83 (2007) 1039–1046. doi:10.1002/jbm.a.31431.

[169] M. Lei, X. Wang, Biodegradable polymers and stem cells for bioprinting, Molecules. 21 (2016) 1–14. doi:10.3390/molecules21050539.

[170] F.-Y. Hsu, M.-R. Lu, R.-C. Weng, H.-M. Lin, Hierarchically biomimetic scaffold of a collagen-mesoporous bioactive glass nanofiber composite for bone tissue engineering., Biomed. Mater. 10 (2015) 25007. doi:10.1088/1748-6041/10/2/025007.

[171] K. Bootsma, M.M. Fitzgerald, B. Free, E. Dimbath, J. Conjerti, G. Reese, D. Konkolewicz, J.A. Berberich, J.L. Sparks, 3D printing of an interpenetrating network hydrogel material with tunable viscoelastic properties, J. Mech. Behav. Biomed. Mater. (2016) 1–11. doi:10.1016/j.jmbbm.2016.07.020.

[172] J. Melke, S. Midha, S. Ghosh, K. Ito, S. Hofmann, Silk fibroin as biomaterial for bone tissue engineering, Acta Biomater. 31 (2016) 1–16. doi:10.1016/j.actbio.2015.09.005.

[173] F. Zhu, L. Cheng, J. Yin, Z.L. Wu, J. Qian, J. Fu, Q. Zheng, 3D Printing of Ultratough Polyion Complex Hydrogels, ACS Appl. Mater. Interfaces. 8 (2016) 31304–31310. doi:10.1021/acsami.6b09881.

[174] D. Chimene, K.K. Lennox, R.R. Kaunas, A.K. Gaharwar, Advanced Bioinks for 3D Printing: A Materials Science Perspective, Ann. Biomed. Eng. 44 (2016) 2090–2102. doi:10.1007/s10439-016-1638-y.

[175] A.R. Short, D. Koralla, A. Deshmukh, B. Wissel, B. Stocker, M. Calhoun, D. Dean, J.O. Winter, Hydrogels that allow and facilitate bone repair, remodeling, and regeneration, J. Mater. Chem. B. 3 (2015) 7818–7830. doi:10.1039/C5TB01043H.

[176] L. Ouyang, C.B. Highley, W. Sun, J.A. Burdick, A Generalizable Strategy for the 3D Bioprinting of Hydrogels from Nonviscous Photo-crosslinkable Inks, Adv. Mater. (2016) 1604983. doi:10.1002/adma.201604983.

[177] G. Gao, T. Yonezawa, K. Hubbell, G. Dai, X. Cui, Inkjet-bioprinted acrylated peptides and PEG hydrogel with human mesenchymal stem cells promote robust bone and cartilage formation with

minimal printhead clogging, Biotechnol. J. 10 (2015) 1568–1577. doi:10.1002/biot.201400635.

[178] H.-W. Kang, S.J. Lee, I.K. Ko, C. Kengla, J.J. Yoo, A. Atala, A 3D bioprinting system to produce human-scale tissue constructs with structural integrity, Nat. Biotechnol. 34 (2016) 312–319. doi:10.1038/nbt.3413.

[179] A.L. Butcher, G.S. Offeddu, M.L. Oyen, Nanofibrous hydrogel composites as mechanically robust tissue engineering scaffolds, Trends Biotechnol. 32 (2014) 564–570. doi:10.1016/j.tibtech.2014.09.001.

[180] K. Chatterjee, S. Lin-Gibson, W.E. Wallace, S.H. Parekh, Y.J. Lee, M.T. Cicerone, M.F. Young, C.G. Simon, The effect of 3D hydrogel scaffold modulus on osteoblast differentiation and mineralization revealed by combinatorial screening, Biomaterials. 31 (2010) 5051–5062. doi:10.1016/j.biomaterials.2010.03.024.

[181] D.M.R. Gibbs, C.R.M. Black, J.I. Dawson, R.O.C. Oreffo, A review of hydrogel use in fracture healing and bone regeneration, J. Tissue Eng. Regen. Med. 10 (2016) 187–198. doi:10.1002/term.1968.

[182] G.D. Nicodemus, S.J. Bryant, Cell encapsulation in biodegradable hydrogels for tissue engineering applications, Tissue Eng. Part B. Rev. 14 (2008) 149–165. doi:10.1089/ten.teb.2007.0332.

[183] A.J. Salgado, O.P. Coutinho, R.L. Reis, Bone tissue engineering: State of the art and future trends, Macromol. Biosci. 4 (2004) 743–765. doi:10.1002/mabi.200400026.

[184] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials. 26 (2005) 5474–5491. doi:10.1016/j.biomaterials.2005.02.002.

[185] M. Dadsetan, T.E. Hefferan, J.P. Szatkowski, P.K. Mishra, S.I. Macura, L. Lu, M.J. Yaszemski, Effect of hydrogel porosity on marrow stromal cell phenotypic expression, Biomaterials. 29 (2008) 2193–2202. doi:10.1016/j.biomaterials.2008.01.006.

[186] P.Y. Huri, B.A. Ozilgen, D.L. Hutton, W.L. Grayson, Scaffold pore size modulates in vitro osteogenesis of human adipose-derived stem/stromal cells., Biomed. Mater. 9 (2014) 45003. doi:10.1088/1748-6041/9/4/045003.

[187] L. Wang, S. Lu, J. Lam, F.K. Kasper, A.G. Mikos, Fabrication of cell-laden macroporous biodegradable hydrogels with tunable porosities and pore sizes., Tissue Eng. Part C. Methods. 21 (2015) 263–73. doi:10.1089/ten.TEC.2014.0224.

[188] D. MacAya, K.K. Ng, M. Spector, Injectable collagen-genipin gel for the treatment of spinal cord injury: In vitro studies, Adv. Funct. Mater. 21 (2011) 4788–4797. doi:10.1002/adfm.201101720.

[189] S.R. Caliari, J.A. Burdick, A practical guide to hydrogels for cell culture., Nat. Methods. 13 (2016) 405–14. doi:10.1038/nmeth.3839.

[190] R.T. Tran, D. Gyawali, Biodegradable Injectable Systems for Bone Tissue Engineering, in: A Handb. Appl. Biopolym. Technol. Synth. Degrad. Appl., 2011: pp. 419–451. doi:10.1039/9781849733458-00419.

[191] Z. Ren, Y. Wang, S. Ma, S. Duan, X. Yang, P. Gao, X. Zhang, Q. Cai, Effective Bone Regeneration Using Thermosensitive Poly(N-Isopropylacrylamide) Grafted Gelatin as Injectable Carrier for Bone Mesenchymal Stem Cells, ACS Appl. Mater. Interfaces. 7 (2015) 19006–19015. doi:10.1021/acsami.5b02821.

[192] I.M.A. Diniz, C. Chen, X. Xu, S. Ansari, H.H. Zadeh, M.M. Marques, S. Shi, A. Moshaverinia, Pluronic F-127 hydrogel as a promising scaffold for encapsulation of dental-derived mesenchymal stem cells, J. Mater. Sci. Mater. Med. 26 (2015) 1–10. doi:10.1007/s10856-015-5493-4.

[193] C.R. Nuttelman, M.C. Tripodi, K.S. Anseth, Synthetic hydrogel niches that promote hMSC viability, Matrix Biol. 24 (2005) 208–218. doi:10.1016/j.matbio.2005.03.004.

[194] A.M. Ferreira, P. Gentile, V. Chiono, G. Ciardelli, Collagen for bone tissue regeneration, Acta Biomater. 8 (2012) 3191–3200. doi:10.1016/j.actbio.2012.06.014.

[195] X. Wang, C. Yan, K. Ye, Y. He, Z. Li, J. Ding, Effect of RGD nanospacing on differentiation of stem cells, Biomaterials. 34 (2013) 2865–2874. doi:10.1016/j.biomaterials.2013.01.021.

[196] X. Wang, K. Ye, Z. Li, C. Yan, J. Ding, Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanospacings., Organogenesis. 9 (2013) 280–6. doi:10.4161/org.26080.

[197] E. Nyberg, C. Holmes, T. Witham, W.L. Grayson, Growth factor-eluting technologies for bone tissue engineering, Drug Deliv. Transl. Res. 6 (2016) 184–194. doi:10.1007/s13346-015-0233-3.

[198] a Aravamudhan, D.M. Ramos, J. Nip, a Subramanian, R. James, M.D. Harmon, X. Yu, S.G. Kumbar, Osteoinductive small molecules: growth factor alternatives for bone tissue engineering, Curr Pharm Des. 19 (2013) 3420–3428. doi:10.2174/1381612811319190008.

[199] T.N. Vo, F.K. Kasper, A.G. Mikos, Strategies for controlled delivery of growth factors and cells for bone regeneration, Adv. Drug Deliv. Rev. 64 (2012) 1292–1309. doi:10.1016/j.addr.2012.01.016.

[200] J. Parker, N. Mitrousis, M.S. Shoichet, Hydrogel for Simultaneous Tunable Growth Factor Delivery and Enhanced Viability of Encapsulated Cells in Vitro, Biomacromolecules. 17 (2016) 476–484. doi:10.1021/acs.biomac.5b01366.

[201] J. Zhang, N. Wang, W. Liu, X. Zhao, W. Lu, Intermolecular hydrogen bonding strategy to fabricate mechanically strong hydrogels with high elasticity and fatigue resistance, Soft Matter. 9 (2013) 6331. doi:10.1039/c3sm50866h.

[202] J.A. Burdick, K.S. Anseth, Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering, Biomaterials. 23 (2002) 4315–4323. doi:10.1016/S0142-9612(02)00176-X.

[203] O. Jeon, K.H. Bouhadir, J.M. Mansour, E. Alsberg, Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties, Biomaterials. 30 (2009) 2724–2734. doi:10.1016/j.biomaterials.2009.01.034.

[204] A.S. Hoffman, Hydrogels for biomedical applications, Adv. Drug Deliv. Rev. 64 (2012) 18–23. doi:10.1016/j.addr.2012.09.010.

[205] F. Lee, K.H. Bae, M. Kurisawa, Injectable hydrogel systems crosslinked by horseradish peroxidase, Biomed. Mater. 11 (2015) 14101. doi:10.1088/1748-6041/11/1/014101.

[206] D.A. Wahl, E. Sachlos, C. Liu, J.T. Czernuszka, Controlling the processing of collagen-hydroxyapatite scaffolds for bone tissue engineering, J. Mater. Sci. Mater. Med. 18 (2007) 201–209. doi:10.1007/s10856-006-0682-9.

[207] P. Giannoudis, C. Tzioupis, T. Almalki, R. Buckley, Fracture healing in osteoporotic fractures: Is it really different?. A basic science perspective, in: N Ashammakhi (Ed.), Injury, 2007: pp. 1–26. doi:10.1016/j.injury.2007.02.014.

[208] O. Snir, J. Bäcklund, J. Boström, I. Andersson, J. Kihlberg, J.H. Buckner, L. Klareskog, R. Holmdahl, V. Malmström, Multifunctional T cell reactivity with native and glycosylated type II collagen in rheumatoid arthritis, Arthritis Rheum. 64 (2012) 2482–2488. doi:10.1002/art.34459.

[209] M.J. Cardoso, R.R. Costa, J.F. Mano, Marine origin polysaccharides in drug delivery systems, Mar. Drugs. 14 (2016) 34. doi:10.3390/md14020034.

[210] R. Logithkumar, A. Keshavnarayan, S. Dhivya, A. Chawla, S. Saravanan, N. Selvamurugan, A review of chitosan and its derivatives in bone tissue engineering, Carbohydr. Polym. 151 (2016) 172–188. doi:10.1016/j.carbpol.2016.05.049.

[211] M. Grellier, P.L. Granja, J.C. Fricain, S.J. Bidarra, M. Renard, R. Bareille, C. Bourget, J. Amédée, M.A. Barbosa, The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect, Biomaterials. 30 (2009) 3271–3278. doi:10.1016/j.biomaterials.2009.02.033.

[212] F. Ahmadi, Z. Oveisi, M. Samani, Z. Amoozgar, Chitosan based hydrogels: Characteristics and pharmaceutical applications, Res. Pharm. Sci. 10 (2015) 1–16.

[213] S.K.L. Levengood, M. Zhang, Chitosan-based scaffolds for bone tissue engineering, J. Mater. Chem. B. 2 (2014) 3161. doi:10.1039/c4tb00027g.

[214] C. Wenling, Effects of the Degree of Deacetylation on the Physicochemical Properties and Schwann Cell Affinity of Chitosan Films, J. Biomater. Appl. 20 (2005) 157–177. doi:10.1177/0885328205049897.

[215] B. Ding, H. Gao, J. Song, Y. Li, L. Zhang, X. Cao, M. Xu, J. Cai, Tough and Cell-compatible Chitosan Physical Hydrogels for Mouse Bone Mesenchymal Stem Cells In Vitro Tough and Cell-compatible Chitosan Physical Hydrogels for Mouse Bone Mesenchymal Stem Cells In Vitro, ACS Appl. Mater. Interfaces. 8 (2016) 19739–46. doi:10.1021/acsami.6b05302.

[216] F. Gao, D. Li, C.H. Bi, Z.H. Mao, B. Adhikari, Preparation and characterization of starch crosslinked with sodium trimetaphosphate and hydrolyzed by enzymes, Carbohydr. Polym. 103 (2014) 310–318. doi:10.1016/j.carbpol.2013.12.028.

[217] Amrita, A. Arora, P. Sharma, D.S. Katti, Pullulan-based composite scaffolds for bone tissue engineering: Improved osteoconductivity by pore wall mineralization, Carbohydr. Polym. 123 (2015) 180–189. doi:10.1016/j.carbpol.2015.01.038.

[218] H. Aydogdu, D. Keskin, E.T. Baran, A. Tezcaner, Pullulan microcarriers for bone tissue regeneration, Mater. Sci. Eng. C. 63 (2016) 439–449. doi:10.1016/j.msec.2016.03.002.

[219] J. Zhu, R.E. Marchant, Design properties of hydrogel tissue-engineering scaffolds., Expert Rev. Med. Devices. 8 (2011) 607–26. doi:10.1586/erd.11.27.

[220] J. Zhu, Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering, Biomaterials. 31 (2010) 4639–4656. doi:10.1016/j.biomaterials.2010.02.044.

[221] P. Huang, X. Bi, J. Gao, L. Sun, S. Wang, S. Chen, X. Fan, Z. You, Y. Wang, Phosphorylated poly(sebacoyl diglyceride) – a phosphate functionalized biodegradable polymer for bone tissue engineering, J. Mater. Chem. B. 4 (2016) 2090–2101. doi:10.1039/C5TB02542G.

[222] N.A. Peppas, K.B. Keys, M. Torres-Lugo, A.M. Lowman, Poly(ethylene glycol)-containing hydrogels in drug delivery, J. Control. Release. 62 (1999) 81–87. doi:10.1016/S0168-3659(99)00027-9.

[223] A.J. García, PEG-maleimide hydrogels for protein and cell delivery in regenerative medicine, Ann. Biomed. Eng. 42 (2014) 312–322. doi:10.1007/s10439-013-0870-y.

[224] C.R. Nuttelman, M.C. Tripodi, K.S. Anseth, In vitro osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels, J. Biomed. Mater. Res. Part A. 68A (2004) 773–782. doi:10.1002/jbm.a.20112.

[225] F. Yang, C.G. Williams, D.A. Wang, H. Lee, P.N. Manson, J. Elisseeff, The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells, Biomaterials. 26 (2005) 5991–5998. doi:10.1016/j.biomaterials.2005.03.018.

[226] M. Elluru, H. Ma, M. Hadjiargyrou, B.S. Hsiao, B. Chu, Synthesis and characterization of biocompatible hydrogel using Pluronics-based block copolymers, Polym. (United Kingdom). 54 (2013) 2088–2095. doi:10.1016/j.polymer.2013.02.017.

[227] G. Niu, F. Du, L. Song, H. Zhang, J. Yang, H. Cao, Y. Zheng, Z. Yang, G. Wang, H. Yang, S. Zhu, Synthesis and characterization of reactive poloxamer 407s for biomedical applications, J. Control. Release. 138 (2009) 49–56. doi:10.1016/j.jconrel.2009.04.026.

[228] J.Y. Jang, S.H. Park, J.H. Park, B.K. Lee, J.H. Yun, B. Lee, J.H. Kim, B.H. Min, M.S. Kim, In Vivo Osteogenic Differentiation of Human Dental Pulp Stem Cells Embedded in an Injectable In Vivo-Forming Hydrogel, Macromol. Biosci. 16 (2016) 1158–1169. doi:10.1002/mabi.201600001.

[229] M.S. Kim, D. Ph, S.U.N.K. Kim, S.H.E.E. Kim, H. Hyun, G. Khang, H.A.I.B. Lee, D. Ph, K.I.M.E.T. Al, In Vivo Osteogenic Differentiation of Rat Bone Marrow Stromal Cells in Thermosensitive MPEG–PCL Diblock Copolymer Gels, Tissue Eng. 12 (2006) 2863–73. doi:10.1089/ten.2006.12.2863.

[230] J.Z. Du, T.M. Sun, S.Q. Weng, X.S. Chen, J. Wang, Synthesis and characterization of photo-cross-linked hydrogels based on biodegradable polyphosphoesters and poly(ethylene glycol) copolymers, Biomacromolecules. 8 (2007) 3375–3381. doi:10.1021/bm700474b.

[231] Q. Li, J. Wang, S. Shahani, D.D.N. Sun, B. Sharma, J.H. Elisseeff, K.W. Leong, Biodegradable and photocrosslinkable polyphosphoester hydrogel, Biomaterials. 27 (2006) 1027–1034. doi:10.1016/j.biomaterials.2005.07.019.

[232] D.A. Wang, C.G. Williams, F. Yang, N. Cher, H. Lee, J.H. Elisseeff, Bioresponsive phosphoester hydrogels for bone tissue engineering, Tissue Eng. 11 (2005) 201–213. doi:10.1089/ten.2005.11.201.

[233] K. Hamada, M. Hirose, T. Yamashita, H. Ohgushi, Spatial distribution of mineralized bone matrix produced by marrow mesenchymal stem cells in self-assembling peptide hydrogel scaffold, J. Biomed. Mater. Res. - Part A. 84 (2008) 128–136. doi:10.1002/jbm.a.31439.

[234] Y. Li, M. Qin, Y. Cao, W. Wang, Designing the mechanical properties of peptide-based supramolecular hydrogels for biomedical applications, Sci. China Physics, Mech. Astron. 57 (2014) 849–858. doi:10.1007/s11433-014-5427-z.

[235] M.A. Khalily, M. Goktas, M.O. Guler, H. Cui, M.J. Webber, S.I. Stupp, R. V. Ulijn, a. M. Smith, a. Dasgupta, J.H. Mondal, D. Das, H. Hosseinkhani, P.D. Hong, D.S. Yu, a. M. Jonker, D.W.P.M. Lowik, J.C.M. Van Hest, S. Bhattacharjee, S. Bhattacharya, P. Moitra, K. Kumar, P. Kondaiah, S. Bhattacharya, X. Zhao, F. Pan, H. Xu, M. Yaseen, H. Shan, C. a. Hauser, S. Zhang, J.R. Lu, M. a. Khalily, O. Ustahuseyin, R. Garifullin, R. Genc, M.O. Guler, M.O. Guler, S.I. Stupp, S.K.M. Nalluri, C. Berdugo, N. Javid, P.W.J.M. Frederix, R. V. Ulijn, Y. Ding, Y. Li, M. Qin, Y. Cao, W. Wang, J.C. Stendahl, M.S. Rao, M.O. Guler, S.I. Stupp, W.Y. Seow, C. a. E. Hauser, J. Boekhoven, J.M. Poolman, C. Maity, F. Li, L. Van Der Mee, C.B. Minkenberg, E. Mendes, J.H. Van Esch, R.

Eelkema, Y. Li, Y. Ding, M. Qin, Y. Cao, W. Wang, M.E. Belowich, J.F. Stoddart, L.H.H.O. Damink, P.J. Dijkstra, M.J. a. Vanluyn, P.B. Vanwachem, P. Nieuwenhuis, J. Feijen, I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, J.H. Bowes, C.W. Cater, Tuning viscoelastic properties of supramolecular peptide gels via dynamic covalent crosslinking, Org. Biomol. Chem. 13 (2015) 1983–1987. doi:10.1039/C4OB02217C.

[236] R.K. Schneider, A. Puellen, R. Kramann, K. Raupach, J. Bornemann, R. Knuechel, A. Pérez-Bouza, S. Neuss, The osteogenic differentiation of adult bone marrow and perinatal umbilical mesenchymal stem cells and matrix remodelling in three-dimensional collagen scaffolds, Biomaterials. 31 (2010) 467–480. doi:10.1016/j.biomaterials.2009.09.059.

[237] J. Venkatesan, I. Bhatnagar, P. Manivasagan, K. Kang, S. Kim, Alginate composites for bone tissue engineering: A review., Int. J. Biol. Macromol. 72C (2014) 269–281. doi:10.1016/j.ijbiomac.2014.07.008.

[238] D. Gothard, E.L. Smith, J.M. Kanczler, C.R. Black, J.A. Wells, C.A. Roberts, L.J. White, O. Qutachi, H. Peto, H. Rashidi, L. Rojo, M.M. Stevens, A.J. El Haj, F.A.R.J. Rose, K.M. Shakesheff, R.O.C. Oreffo, In vivo assessment of bone regeneration in alginate/bone ECM hydrogels with incorporated skeletal stem cells and single growth factors, PLoS One. 10 (2015) e0145080. doi:10.1371/journal.pone.0145080.

[239] H. Liu, H. Peng, Y. Wu, C. Zhang, Y. Cai, G. Xu, Q. Li, X. Chen, J. Ji, Y. Zhang, H.W. OuYang, The promotion of bone regeneration by nanofibrous hydroxyapatite/chitosan scaffolds by effects on integrin-BMP/Smad signaling pathway in BMSCs, Biomaterials. 34 (2013) 4404–4417. doi:10.1016/j.biomaterials.2013.02.048.

[240] C. Hayashi, U. Hasegawa, Y. Saita, H. Hemmi, T. Hayata, K. Nakashima, Y. Ezura, T. Amagasa, K. Akiyoshi, M. Noda, Osteoblastic bone formation is induced by using nanogel-crosslinking hydrogel as novel scaffold for bone growth factor, J. Cell. Physiol. 220 (2009) 1–7. doi:10.1002/jcp.21760.

[241] J.A. Hunt, R. Chen, T. van Veen, N. Bryan, Hydrogels for tissue engineering and regenerative medicine, J. Mater. Chem. B. 2 (2014) 5319–5338. doi:10.1039/C4TB00775A.

[242] M.B. Dreifke, N.A. Ebraheim, A.C. Jayasuriya, Investigation of potential injectable polymeric biomaterials for bone regeneration, J. Biomed. Mater. Res. - Part A. 101 A (2013) 2436–2447. doi:10.1002/jbm.a.34521.

[243] B.M. Watson, F.K. Kasper, A.G. Mikos, Phosphorous-containing polymers for regenerative medicine., Biomed. Mater. 9 (2014) 25014. doi:10.1088/1748-6041/9/2/025014.

[244] H. Nakahara, H. Misawa, A. Yoshida, T. Hayashi, M. Tanaka, T. Furumatsu, N. Tanaka, N. Kobayashi, T. Ozaki, Bone repair using a hybrid scaffold of self-assembling peptide PuraMatrix and polyetheretherketone cage in rats, Cell Transplant. 19 (2010) 791–797. doi:10.3727/096368910X508906.

[245] H. Misawa, N. Kobayashi, A. Soto-Gutierrez, Y. Chen, A. Yoshida, J.D. Rivas-Carrillo, N. Navarro-Alvarez, K. Tanaka, A. Miki, J. Takei, T. Ueda, M. Tanaka, H. Endo, N. Tanaka, T. Ozaki, PuraMatrix facilitates bone regeneration in bone defects of calvaria in mice, Cell Transplant. 15 (2006) 903–910. doi:10.3727/000000006783981369.

[246] D.J. Munoz-Pinto, A.C. Jimenez-Vergara, T.P. Gharat, M.S. Hahn, Characterization of sequential collagen-poly(ethylene glycol) diacrylate interpenetrating networks and initial assessment of their potential for vascular tissue engineering, Biomaterials. 40 (2015) 32–42. doi:10.1016/j.biomaterials.2014.10.051.

[247] D. Cross, X. Jiang, W. Ji, W. Han, C. Wang, Injectable hybrid hydrogels of hyaluronic acid crosslinked by well-defined synthetic polycations: Preparation and characterization in vitro and in vivo, Macromol. Biosci. 15 (2015) 668–681. doi:10.1002/mabi.201400491.

[248] S. Frasca, F. Norol, C. Le Visage, J.-M. Collombet, D. Letourneur, X. Holy, E. Sari Ali, Calcium-phosphate ceramics and polysaccharide-based hydrogel scaffolds combined with mesenchymal stem cell differently support bone repair in rats, J. Mater. Sci. Mater. Med. 28 (2017) 35. doi:10.1007/s10856-016-5839-6.

[249] K. Na, S. won Kim, B.K. Sun, D.G. Woo, H.N. Yang, H.M. Chung, K.H. Park, Osteogenic differentiation of rabbit mesenchymal stem cells in thermo-reversible hydrogel constructs containing hydroxyapatite and bone morphogenic protein-2 (BMP-2), Biomaterials. 28 (2007) 2631–2637. doi:10.1016/j.biomaterials.2007.02.008.

[250] H. Vihola, A. Laukkanen, L. Valtola, H. Tenhu, J. Hirvonen, Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam), Biomaterials. 26 (2005) 3055–3064. doi:10.1016/j.biomaterials.2004.09.008.

[251] A.L. Barnes, P.G. Genever, S. Rimmer, M.C. Coles, Collagen-Poly(N-isopropylacrylamide) Hydrogels with Tunable Properties, Biomacromolecules. 17 (2016) 723–734. doi:10.1021/acs.biomac.5b01251.

[252] J.H. Cho, S.-H. Kim, K.D. Park, M.C. Jung, W.I. Yang, S.W. Han, J.Y. Noh, J.W. Lee, Chondrogenic differentiation of human mesenchymal stem cells using a thermosensitive poly(N-isopropylacrylamide) and water-soluble chitosan copolymer., Biomaterials. 25 (2004) 5743–5751. doi:10.1016/j.biomaterials.2004.01.051.

[253] K. Na, S. Kim, D.G. Woo, B.K. Sun, H.N. Yang, H.M. Chung, K.H. Park, Synergistic effect of TGFβ-3 on chondrogenic differentiation of rabbit chondrocytes in thermo-reversible hydrogel constructs

*

blended with hyaluronic acid by in vivo test, J. Biotechnol. 128 (2007) 412–422. doi:10.1016/j.jbiotec.2006.09.025.

[254] E. Smith, J. Yang, L. McGann, W. Sebald, H. Uludag, RGD-grafted thermoreversible polymers to facilitate attachment of BMP-2 responsive C2C12 cells, Biomaterials. 26 (2005) 7329–7338. doi:10.1016/j.biomaterials.2005.05.060.

[255] H.-T. Liao, C.-T. Chen, J.-P. Chen, Osteogenic Differentiation and Ectopic Bone Formation of Canine Bone Marrow-Derived Mesenchymal Stem Cells in Injectable Thermo-Responsive Polymer Hydrogel, Tissue Eng. Part C Methods. 17 (2011) 1139–1149. doi:10.1089/ten.tec.2011.0140.

[256] F. Baino, G. Novajra, C. Vitale-Brovarone, Bioceramics and Scaffolds: A Winning Combination for Tissue Engineering., Front. Bioeng. Biotechnol. 3 (2015) 202. doi:10.3389/fbioe.2015.00202.

[257] J. Xie, M.J. Baumann, L.R. McCabe, Osteoblasts respond to hydroxyapatite surfaces with immediate changes in gene expression, J. Biomed. Mater. Res. - Part A. 71 (2004) 108–117. doi:10.1002/jbm.a.30140.

[258] D.A. Wahl, J.T. Czernuszka, Collagen-hydroxyapatite composites for hard tissue repair, Eur. Cells Mater. 11 (2006) 43–56. doi:vol011a06 [pii].

[259] G. Turco, E. Marsich, F. Bellomo, S. Semeraro, I. Donati, F. Brun, M. Grandolfo, A. Accardo, S.

Paoletti, Alginate/hydroxyapatite biocomposite for bone ingrowth: A trabecular structure with high and isotropic connectivity, Biomacromolecules. 10 (2009) 1575–1583. doi:10.1021/bm900154b.

[260] F. Zhao, D. Yao, R. Guo, L. Deng, A. Dong, J. Zhang, Composites of Polymer Hydrogels and Nanoparticulate Systems for Biomedical and Pharmaceutical Applications, Nanomaterials. 5 (2015) 2054–2130. doi:10.3390/nano5042054.

[261] D.N. Heo, N. Castro, S.-J. Lee, H. Noh, W. Zhu, L.G. Zhang, Enhanced bone tissue regeneration using 3D printed microstructure incorporated with hybrid nano hydrogel, Nanoscale. 16 (2017) 3819–3826. doi:10.1039/C6NR09652B.

[262] PubMed, https://www.ncbi.nlm.nih.gov/pubmed/, (n.d.).

[263] ClinicalTrials.gov, https://ClinicalTrials.gov/, (n.d.).

[264] R. Dai, Z. Wang, R. Samanipour, K. Koo, K. Kim, Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications, Stem Cells Int. 2016 (2016) 1–19. doi:10.1155/2016/6737345.

[265] L. Frese, P.E. Dijkman, S.P. Hoerstrup, Adipose tissue-derived stem cells in regenerative medicine, Transfus. Med. Hemotherapy. 43 (2016) 268–274. doi:10.1159/000448180.

[266] A. Pantalone, I. Antonucci, M. Guelfi, P. Pantalone, Amniotic fluid stem cells : an ideal resource for therapeutic application in bone tissue engineering, Eur. Rev. Med. Pharmacol. Sci. (2016) 1–7.

[267] A.M. Yousefi, P.F. James, R. Akbarzadeh, A. Subramanian, C. Flavin, H. Oudadesse, Prospect of stem cells in bone tissue engineering: A review, Stem Cells Int. 2016 (2016) 6180487. doi:10.1155/2016/6180487.

[268] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement., Cytotherapy. 8 (2006) 315–7. doi:10.1080/14653240600855905.

[269] K.C. Elahi, G. Klein, M. Avci-Adali, K.D. Sievert, S. Macneil, W.K. Aicher, Human mesenchymal stromal cells from different sources diverge in their expression of cell surface proteins and display distinct differentiation patterns, Stem Cells Int. 2016 (2016) 5646384. doi:10.1155/2016/5646384.

[270] J.S. Heo, Y. Choi, H.O.H.-S.H. Kim, H.O.H.-S.H. Kim, Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue., Int. J. Mol. Med. 37 (2015) 115–125. doi:10.3892/ijmm.2015.2413.

[271] D.H. Woo, H.S. Hwang, J.H. Shim, Comparison of adult stem cells derived from multiple stem cell niches, Biotechnol. Lett. 38 (2016) 751–759. doi:10.1007/s10529-016-2050-2.

[272] V. Guneta, N.S. Tan, S.K.J. Chan, V. Tanavde, T.C. Lim, T.C.M. Wong, C. Choong, Comparative study of adipose-derived stem cells and bone marrow-derived stem cells in similar microenvironmental conditions, Exp. Cell Res. 348 (2016) 155–164. doi:10.1016/j.yexcr.2016.09.012.

[273] J. Jensen, C. Tvedesøe, J.H.D. Rölfing, C.B. Foldager, H. Lysdahl, D.C.E. Kraft, M. Chen, J. Baas, D.Q.S. Le, C.E. Bünger, Dental pulp-derived stromal cells exhibit a higher osteogenic potency than bone marrow-derived stromal cells in vitro and in a porcine critical-size bone defect model., Sicot-J. 2 (2016) 16. doi:10.1051/sicotj/2016004.

[274] W.J. Du, Y. Chi, Z.X. Yang, Z.J. Li, J.J. Cui, B.Q. Song, X. Li, S.G. Yang, Z.B. Han, Z.C. Han,

Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta., Stem Cell Res. Ther. 7 (2016) 163. doi:10.1186/s13287-016-0418-9.

[275] D. Blashki, M.B. Murphy, M. Ferrari, P.J. Simmons, E. Tasciotti, Mesenchymal stem cells from cortical bone demonstrate increased clonal incidence, potency, and developmental capacity compared to their bone marrow-derived counterparts., J. Tissue Eng. 7 (2016) 2041731416661196. doi:10.1177/2041731416661196.

[276] S.J. Roberts, N. van Gastel, G. Carmeliet, F.P. Luyten, Uncovering the periosteum for skeletal regeneration: The stem cell that lies beneath, Bone. 70 (2015) 10–18. doi:10.1016/j.bone.2014.08.007.

[277] E. Jones, R. Schafer, Where is the common ground between bone marrow mesenchymal stem/stromal cells from different donors and species?, Stem Cell Res Ther. 6 (2015) 143. doi:10.1186/s13287-015-0144-8.

[278] M. Maredziak, K. Marycz, K.A. Tomaszewski, K. Kornicka, B.M. Henry, M. Marędziak, K. Marycz, K.A. Tomaszewski, K. Kornicka, B.M. Henry, The Influence of Aging on the Regenerative Potential of Human Adipose Derived Mesenchymal Stem Cells, Stem Cells Int. 2016 (2016) 1–15. doi:10.1155/2016/2152435.

[279] V. Pérez-Silos, A. Camacho-Morales, L. Fuentes-Mera, Mesenchymal Stem Cells Subpopulations: Application for Orthopedic Regenerative Medicine, Stem Cells Int. 2016 (2016) 1–9. doi:10.1155/2016/3187491.

[280] E.R. Zielins, R. Tevlin, M.S. Hu, M.T. Chung, A. McArdle, K.J. Paik, D. Atashroo, C.R. Duldulao, A. Luan, K. Senarath-Yapa, G.G. Walmsley, T. Wearda, M.T. Longaker, D.C. Wan, Isolation and enrichment of human adipose-derived stromal cells for enhanced osteogenesis., J. Vis. Exp. (2015) 52181. doi:10.3791/52181.

[281] M.A. König, D.D. Canepa, D. Cadosch, E. Casanova, M. Heinzelmann, D. Rittirsch, M. Plecko, S. Hemmi, H.-P.P. Simmen, P. Cinelli, G.A. Wanner, M.A. König, D.D. Canepa, D. Cadosch, E. Casanova, M. Heinzelmann, D. Rittirsch, M. Plecko, S. Hemmi, H.-P.P. Simmen, P. Cinelli, G.A. Wanner, Direct transplantation of native pericytes from adipose tissue: A new perspective to stimulate healing in critical size bone defects, Cytotherapy. 18 (2016) 41–52. doi:10.1016/j.jcyt.2015.10.002.

[282] M. Maniscalco, A. Bianco, G. Mazzarella, A. Motta, Recent Advances on Nitric Oxide in the Upper Airways., Curr. Med. Chem. 23 (2016) 2736–2745. doi:10.2174/0929867323666160627.

[283] P. Giannoni, S. Scaglione, A. Daga, C. Ilengo, M. Cilli, R. Quarto, Short-time survival and engraftment of bone marrow stromal cells in an ectopic model of bone regeneration., Tissue Eng. Part A. 16 (2010) 489–499. doi:10.1089/ten.tea.2009.0041.

[284] H. Agrawal, H. Shang, A. Sattah, N. Yang, S. Peirce, A. Katz, Human adipose-derived stromal/stem cells demonstrate short-lived persistence after implantation in both an immunocompetent and an immunocompromised murine model, Stem Cell Res. Ther. 5 (2014) 142. doi:10.1186/scrt532.

[285] S. Lee, E. Choi, M.J. Cha, K.C. Hwang, Cell adhesion and long-term survival of transplanted mesenchymal stem cells: A prerequisite for cell therapy, Oxid. Med. Cell. Longev. 2015 (2015) 1–9. doi:10.1155/2015/632902.

[286] P. Becquart, a Cambon-Binder, L.E. Monfoulet, M. Bourguignon, K. Vandamme, M. Bensidhoum, H. Petite, D. Logeart-Avramoglou, Ischemia Is the Prime but Not the Only Cause of Human

Multipotent Stromal Cell Death in Tissue-Engineered Constructs In Vivo, Tissue Eng Part A. 18 (2012) 2084–2094. doi:10.1089/ten.TEA.2011.0690.

[287] N. Kimelman-Bleich, G. Pelled, D. Sheyn, I. Kallai, Y. Zilberman, O. Mizrahi, Y. Tal, W. Tawackoli, Z. Gazit, D. Gazit, The use of a synthetic oxygen carrier-enriched hydrogel to enhance mesenchymal stem cell-based bone formation in vivo, Biomaterials. 30 (2009) 4639–4648. doi:10.1016/j.biomaterials.2009.05.027.

[288] S. Benjamin, D. Sheyn, S. Ben-David, A. Oh, I. Kallai, N. Li, D. Gazit, Z. Gazit, Oxygenated environment enhances both stem cell survival and osteogenic differentiation., Tissue Eng. Part A. 19 (2013) 748–58. doi:10.1089/ten.TEA.2012.0298.

[289] M. Deschepper, M. Manassero, K. Oudina, J. Paquet, L.E. Monfoulet, M. Bensidhoum, D. Logeart-Avramoglou, H. Petite, Proangiogenic and prosurvival functions of glucose in human mesenchymal stem cells upon transplantation, Stem Cells. 31 (2013) 526–535. doi:10.1002/stem.1299.

[290] M. Deschepper, K. Oudina, B. David, V. Myrtil, C. Collet, M. Bensidhoum, D. Logeart-Avramoglou, H. Petite, Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome exposure to long-term, severe and continuous hypoxia, J. Cell. Mol. Med. 15 (2011) 1505–1514. doi:10.1111/j.1582-4934.2010.01138.x.

[291] J. Lee, A.A. Abdeen, K.A. Kilian, Rewiring mesenchymal stem cell lineage specification by switching the biophysical microenvironment., Sci. Rep. 4 (2014) 5188. doi:10.1038/srep05188.

[292] W.T. Hsieh, Y.S. Liu, Y.H. Lee, M.G. Rimando, K.H. Lin, O.K. Lee, Matrix dimensionality and stiffness cooperatively regulate osteogenesis of mesenchymal stromal cells, Acta Biomater. 32 (2016) 210–222. doi:10.1016/j.actbio.2016.01.010.

[293] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix Elasticity Directs Stem Cell Lineage Specification, Cell. 126 (2006) 677–689. doi:10.1016/j.cell.2006.06.044.

[294] P.J. Stiers, N. van Gastel, G. Carmeliet, Targeting the hypoxic response in bone tissue engineering: A balance between supply and consumption to improve bone regeneration, Mol. Cell. Endocrinol. 432 (2016) 96–105. doi:10.1016/j.mce.2015.12.024.

[295] D. Henrich, C. Nau, S.B. Kraft, M. Zollfrank, K. Kontradowitz, E. Oppermann, J. Schultheiss, S. Meier, J. Frank, I. Marzi, C. Seebach, Effect of the harvest procedure and tissue site on the osteogenic function of and gene expression in human mesenchymal stem cells, Int. J. Mol. Med. 37 (2016) 976–988. doi:10.3892/ijmm.2016.2489.

[296] M.C. Corotchi, M.A. Popa, A. Remes, L.E. Sima, I. Gussi, M.L. Plesu, Isolation method and xeno-free culture conditions influence multipotent differentiation capacity of human Wharton's jelly-derived mesenchymal stem cells, Stem Cell Res. Ther. 4 (2013) 1. doi:10.1186/scrt232.

[297] V. Gatta, M. D'Aurora, P. Lanuti, L. Pierdomenico, S. Sperduti, G. Palka, M. Gesi, M. Marchisio, S. Miscia, L. Stuppia, Gene expression modifications in Wharton's Jelly mesenchymal stem cells promoted by prolonged in vitro culturing., BMC Genomics. 14 (2013) 635. doi:10.1186/1471-2164-14-635.

[298] H. Elkhenany, L. Amelse, M. Caldwell, R. Abdelwahed, M. Dhar, Impact of the source and serial passaging of goat mesenchymal stem cells on osteogenic differentiation potential: implications for bone tissue engineering, J. Anim. Sci. Biotechnol. 7 (2016) 16. doi:10.1186/s40104-016-0074-z.

[299] Y. Shuai, L. Liao, X. Su, Y. Yu, B. Shao, H. Jing, X. Zhang, Z. Deng, Y. Jin, Melatonin treatment improves mesenchymal stem cells therapy by preserving stemness during long-term in vitro

expansion, Theranostics. 6 (2016) 1899–1917. doi:10.7150/thno.15412.

[300] X. L., M. M., Z. L., Spheroid Mesenchymal Stem Cells and Mesenchymal Stem Cell-Derived Microvesicles: Two Potential Therapeutic Strategies, Stem Cells Dev. 25 (2016) 203–213. doi:10.1089/scd.2015.0278.

[301] K.C. Murphy, A.I. Hoch, J.N. Harvestine, D. Zhou, J.K. Leach, Mesenchymal Stem Cell Spheroids Retain Osteogenic Phenotype Through α2β1 Signaling., Stem Cells Transl. Med. 5 (2016) 1229–37. doi:10.5966/sctm.2015-0412.

[302] Z. Gamie, R.J. MacFarlane, A. Tomkinson, A. Moniakis, G.T. Tran, Y. Gamie, A. Mantalaris, E. Tsiridis, Skeletal tissue engineering using mesenchymal or embryonic stem cells: clinical and experimental data., Expert Opin. Biol. Ther. 14 (2014) 1611–39. doi:10.1517/14712598.2014.945414.

[303] J.J. Cunningham, T.M. Ulbright, M.F. Pera, L.H.J. Looijenga, Lessons from human teratomas to guide development of safe stem cell therapies, Nat. Biotechnol. 30 (2012) 849–857. doi:10.1038/nbt.2329.

[304] C.-Y. Fong, K. Gauthaman, A. Bongso, Teratomas from pluripotent stem cells: A clinical hurdle., J. Cell. Biochem. 111 (2010) 769–781. doi:10.1002/jcb.22775.

[305] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors, Cell. 131 (2007) 861–872. doi:10.1016/j.cell.2007.11.019.

[306] F. Bastami, P. Nazeman, H. Moslemi, M. Rezai Rad, K. Sharifi, A. Khojasteh, Induced pluripotent stem cells as a new getaway for bone tissue engineering: A systematic review, Cell Prolif. 50 (2016) 1–29. doi:10.1111/cpr.12321.

[307] B. Levi, J.S. Hyun, D.T. Montoro, D.D. Lo, C.K.F. Chan, S. Hu, N. Sun, M. Lee, M. Grova, A.J. Connolly, J.C. Wu, G.C. Gurtner, I.L. Weissman, D.C. Wan, M.T. Longaker, In vivo directed differentiation of pluripotent stem cells for skeletal regeneration., Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 20379–84. doi:10.1073/pnas.1218052109.

[308] G.M. de Peppo, I. Marcos-Campos, D.J. Kahler, D. Alsalman, L. Shang, G. Vunjak-Novakovic, D. Marolt, Engineering bone tissue substitutes from human induced pluripotent stem cells., Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 8680–5. doi:10.1073/pnas.1301190110.

[309] Q. Wu, B. Yang, K. Hu, C. Cao, Y. Man, P. Wang, Deriving Osteogenic Cells from Induced Pluripotent Stem Cells for Bone Tissue Engineering, Tissue Eng. Part B Rev. 23 (2016) ten.teb.2015.0559. doi:10.1089/ten.teb.2015.0559.

[310] G.M. de Peppo, D. Marolt, Modulating the biochemical and biophysical culture environment to enhance osteogenic differentiation and maturation of human pluripotent stem cell-derived mesenchymal progenitors., Stem Cell Res. Ther. 4 (2013) 106. doi:10.1186/scrt317.

[311] G.M. de Peppo, D. Marolt, Make no bones about it: cells could soon be reprogrammed to grow replacement bones?, Expert Opin Biol Ther. 14 (2014) 1–5. doi:10.1517/14712598.2013.840581.

[312] O.H. Jeon, L.M. Panicker, Q. Lu, J.J. Chae, R.A. Feldman, J.H. Elisseeff, Human iPSC-derived osteoblasts and osteoclasts together promote bone regeneration in 3D biomaterials., Sci. Rep. 6 (2016) 26761. doi:10.1038/srep26761.

[313] J.E. Park, G.A. Keller, N. Ferrara, The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular

matrix-bound VEGF., Mol. Biol. Cell. 4 (1993) 1317–26. doi:10.1091/mbc.4.12.1317.

[314] B. Beckermann, G. Kallifatidis, A. Groth, D. Frommhold, A. Apel, J. Mattern, A. Salnikov, G. Moldenhauer, W. Wagner, A. Diehlmann, R. Saffrich, M. Schubert, A. Ho, N. Giese, M. Bü Chler, H. Friess, P. Bü Chler, I. Herr, VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma, Br. J. Cancer. 99 (2008) 622–631. doi:10.1038/sj.bjc.6604508.

[315] S.S. Ho, K.C. Murphy, B.Y.K. Binder, C.B. Vissers, J.K. Leach, Increased Survival and Function of Mesenchymal Stem Cell Spheroids Entrapped in Instructive Alginate Hydrogels., Stem Cells Transl. Med. 5 (2016) sctm.2015-0211. doi:10.5966/sctm.2015-0211.

[316] X.F. Shi, H. Wang, F.J. Xiao, Y. Yin, Q.Q. Xu, R.L. Ge, L.S. Wang, MiRNA-486 regulates angiogenic activity and survival of mesenchymal stem cells under hypoxia through modulating Akt signal, Biochem. Biophys. Res. Commun. 470 (2016) 670–677. doi:10.1016/j.bbrc.2016.01.084.

[317] A.M. Bader, K. Klose, K. Bieback, D. Korinth, M. Schneider, M. Seifert, Y.H. Choi, A. Kurtz, V. Falk, C. Stamm, Hypoxic preconditioning increases survival and pro-Angiogenic capacity of human cord blood mesenchymal stromal cells in vitro, PLoS One. 10 (2015) e0138477. doi:10.1371/journal.pone.0138477.

[318] D. Gothard, E.L. Smith, J.M. Kanczler, H. Rashidi, O. Qutachi, J. Henstock, M. Rotherham, A. El Haj, K.M. Shakesheff, R.O.C. Oreffo, Tissue engineered bone using select growth factors: A comprehensive review of animal studies and clinical translation studies in man., Eur. Cell. Mater. 28 (2014) 166-207–8. doi:10.22203/eCM.v028a13.

[319] M.M. Martino, S. Brkic, E. Bovo, M. Burger, D.J. Schaefer, T. Wolff, L. Gürke, P.S. Briquez, H.M. Larsson, R. Gianni-Barrera, J.A. Hubbell, A. Banfi, Extracellular matrix and growth factor engineering for controlled angiogenesis in regenerative medicine., Front. Bioeng. Biotechnol. 3 (2015) 45. doi:10.3389/fbioe.2015.00045.

[320] E. Wernike, M.O. Montjovent, Y. Liu, D. Wismeijer, E.B. Hunziker, K.A. Siebenrock, W. Hofstetter, F.M. Klenke, Vegf incorporated into calcium phosphate ceramics promotes vascularisation and bone formation in vivo, Eur. Cells Mater. 19 (2010) 30–40. doi:vol019a04 [pii].

[321] S. Stegen, N. van Gastel, G. Carmeliet, Bringing new life to damaged bone: The importance of angiogenesis in bone repair and regeneration, Bone. 70 (2015) 19–27. doi:10.1016/j.bone.2014.09.017.

[322] K. Sun, Z. Zhou, X. Ju, Y. Zhou, J. Lan, D. Chen, H. Chen, M. Liu, L. Pang, Combined transplantation of mesenchymal stem cells and endothelial progenitor cells for tissue engineering: a systematic review and meta-analysis, Stem Cell Res. Ther. 7 (2016) 151. doi:10.1186/s13287-016-0390-4.

[323] J. Buschmann, M. Welti, S. Hemmi, P. Neuenschwander, C. Baltes, P. Giovanoli, M. Rudin, M. Calcagni, Three-dimensional co-cultures of osteoblasts and endothelial cells in DegraPol foam: histological and high-field magnetic resonance imaging analyses of pre-engineered capillary networks in bone grafts., Tissue Eng. Part A. 17 (2011) 291–299. doi:10.1089/ten.tea.2010.0278.

[324] S. Ghanaati, R.E. Unger, M.J. Webber, M. Barbeck, C. Orth, J.A. Kirkpatrick, P. Booms, A. Motta, C. Migliaresi, R.A. Sader, C.J. Kirkpatrick, Scaffold vascularization in vivo driven by primary human osteoblasts in concert with host inflammatory cells, Biomaterials. 32 (2011) 8150–8160. doi:10.1016/j.biomaterials.2011.07.041.

[325] R.E. Unger, S. Ghanaati, C. Orth, A. Sartoris, M. Barbeck, S. Halstenberg, A. Motta, C. Migliaresi, C.J. Kirkpatrick, The rapid anastomosis between prevascularized networks on silk fibroin scaffolds

generated in vitro with cocultures of human microvascular endothelial and osteoblast cells and the host vasculature, Biomaterials. 31 (2010) 6959–6967. doi:10.1016/j.biomaterials.2010.05.057.

[326] N.B. Thébaud, R. Siadous, R. Bareille, M. Remy, R. Daculsi, J. Amédée, L. Bordenave, Whatever their differentiation status, human progenitor derived - or mature - endothelial cells induce osteoblastic differentiation of bone marrow stromal cells, J. Tissue Eng. Regen. Med. 6 (2012) 51–60. doi:10.1002/term.1539.

[327] J. Guerrero, S. Catros, S.M. Derkaoui, C. Lalande, R. Siadous, R. Bareille, N. Thébaud, L. Bordenave, O. Chassande, C. Le Visage, D. Letourneur, J. Amédée, Cell interactions between human progenitor-derived endothelial cells and human mesenchymal stem cells in a three-dimensional macroporous polysaccharide-based scaffold promote osteogenesis, Acta Biomater. 9 (2013) 8200–8213. doi:10.1016/j.actbio.2013.05.025.

[328] M. Kazemzadeh-Narbat, J. Rouwkema, N. Annabi, H. Cheng, M. Ghaderi, B.-H. Cha, M. Aparnathi, A. Khalilpour, B. Byambaa, E. Jabbari, A. Tamayol, A. Khademhosseini, Engineering Photocrosslinkable Bicomponent Hydrogel Constructs for Creating 3D Vascularized Bone, Adv. Healthc. Mater. (2017) 1601122. doi:10.1002/adhm.201601122.

[329] S. Liu, J. Zhou, X. Zhang, Y. Liu, J. Chen, B. Hu, J. Song, Y. Zhang, Strategies to optimize adult stem cell therapy for tissue regeneration, Int. J. Mol. Sci. 17 (2016) 86–23. doi:10.3390/ijms17060982.

[330] J. Kobolak, A. Dinnyes, A. Memic, A. Khademhosseini, A. Mobasheri, Mesenchymal stem cells: Identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche, Methods. 99 (2016) 62–68. doi:10.1016/j.ymeth.2015.09.016.

[331] V. Agrawal, M. Sinha, A review on carrier systems for bone morphogenetic protein-2, J Biomed Mater Res B Appl Biomater. (2016) 1–22. doi:10.1002/jbm.b.33599.

[332] L. Meinel, O. Betz, R. Fajardo, S. Hofmann, A. Nazarian, E. Cory, M. Hilbe, J. McCool, R. Langer, G. Vunjak-Novakovic, H.P. Merkle, B. Rechenberg, D.L. Kaplan, C. Kirker-Head, Silk based biomaterials to heal critical sized femur defects, Bone. 39 (2006) 922–931. doi:10.1016/j.bone.2006.04.019.

[333] M.W. Laschke, T.E. Schank, C. Scheuer, S. Kleer, T. Shadmanov, D. Eglin, M. Alini, M.D. Menger, In vitro osteogenic differentiation of adipose-derived mesenchymal stem cell spheroids impairs their in vivo vascularization capacity inside implanted porous polyurethane scaffolds, Acta Biomater. 10 (2014) 4226–4235. doi:10.1016/j.actbio.2014.06.035.

[334] J. Guerrero, H. Oliveira, S. Catros, R. Siadous, S.-M. Derkaoui, R. Bareille, D. Letourneur, J. Amédée, The use of total human bone marrow fraction in a direct three-dimensional expansion approach for bone tissue engineering applications: focus on angiogenesis and osteogenesis., Tissue Eng. Part A. 21 (2015) 861–74. doi:10.1089/ten.TEA.2014.0367.

[335] S.S. Ho, N.L. Vollmer, M.I. Refaat, O. Jeon, E. Alsberg, M.A. Lee, J.K. Leach, Bone Morphogenetic Protein-2 Promotes Human Mesenchymal Stem Cell Survival and Resultant Bone Formation When Entrapped in Photocrosslinked Alginate Hydrogels, Adv. Healthc. Mater. 5 (2016) 2501–2509. doi:10.1002/adhm.201600461.

[336] B.B. Seo, H. Choi, J.T. Koh, S.C. Song, Sustained BMP-2 delivery and injectable bone regeneration using thermosensitive polymeric nanoparticle hydrogel bearing dual interactions with BMP-2, J. Control. Release. 209 (2015) 67–76. doi:10.1016/j.jconrel.2015.04.023.

[337] D. Ben-David, S. Srouji, K. Shapira-Schweitzer, O. Kossover, E. Ivanir, G. Kuhn, R. Müller, D.

Seliktar, E. Livne, Low dose BMP-2 treatment for bone repair using a PEGylated fibrinogen hydrogel matrix, Biomaterials. 34 (2013) 2902–2910. doi:10.1016/j.biomaterials.2013.01.035.

[338] A.W. James, G. LaChaud, J. Shen, G. Asatrian, V. Nguyen, X. Zhang, K. Ting, C. Soo, A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2, Tissue Eng. Part B Rev. 22 (2016) ten.TEB.2015.0357. doi:10.1089/ten.TEB.2015.0357.

[339] Y. Il Chung, K.M. Ahn, S.H. Jeon, S.Y. Lee, J.H. Lee, G. Tae, Enhanced bone regeneration with BMP-2 loaded functional nanoparticle-hydrogel complex, J. Control. Release. 121 (2007) 91–99. doi:10.1016/j.jconrel.2007.05.029.

[340] G. Bhakta, B. Rai, Z.X.H. Lim, J.H. Hui, G.S. Stein, A.J. van Wijnen, V. Nurcombe, G.D. Prestwich, S.M. Cool, Hyaluronic acid-based hydrogels functionalized with heparin that support controlled release of bioactive BMP-2, Biomaterials. 33 (2012) 6113–6122. doi:10.1016/j.biomaterials.2012.05.030.

[341] S. Scarfì, Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair, World J. Stem Cells. 8 (2016) 1. doi:10.4252/wjsc.v8.i1.1.

[342] J.-H. Hong, TAZ, a Transcriptional Modulator of Mesenchymal Stem Cell Differentiation, Science (80-.). 309 (2005) 1074–1078. doi:10.1126/science.1110955.

[343] O. Jeon, J.W. Rhie, I.-K. Kwon, J.-H. Kim, B.-S. Kim, S.-H. Lee, In vivo bone formation following transplantation of human adipose-derived stromal cells that are not differentiated osteogenically., Tissue Eng. Part A. 14 (2008) 1285–1294. doi:10.1089/ten.tea.2007.0253.

[344] A. Mehrkens, F. Saxer, S. Güven, W. Hoffmann, A.M. Müller, M. Jakob, F.E. Weber, I. Martin, A. Scherberich, Intraoperative engineering of osteogenic grafts combining freshly harvested, human adipose-derived cells and physiological doses of bone morphogenetic protein-2, Eur. Cells Mater. 24 (2012) 308–319. doi:10.22203/eCM.v024a22.

[345] Y.-F. Chou, P. a Zuk, T.-L. Chang, P. Benhaim, B.M. Wu, Adipose-derived stem cells and BMP2: part 1. BMP2-treated adipose-derived stem cells do not improve repair of segmental femoral defects., Connect. Tissue Res. 52 (2011) 109–118. doi:10.3109/03008207.2010.484514.

[346] S. Kim, S.S. Kim, S.H. Lee, S. Eun Ahn, S.J. Gwak, J.H. Song, B.S. Kim, H.M. Chung, In vivo bone formation from human embryonic stem cell-derived osteogenic cells in poly(d,l-lactic-co-glycolic acid)/hydroxyapatite composite scaffolds, Biomaterials. 29 (2008) 1043–1053. doi:10.1016/j.biomaterials.2007.11.005.

[347] A. Nuschke, M. Rodrigues, J. Rivera, C. Yates, D. Whaley, D. Stolz, L. Griffith, A. Wells, Epidermal Growth Factor Tethered to -Tricalcium Phosphate Bone Scaffolds via a High-Affinity Binding Peptide Enhances Survival of Human Mesenchymal Stem Cells/Multipotent Stromal Cells in an Immune-Competent Parafascial Implantation Assay in Mice, Stem Cells Transl. Med. 5 (2016) 365–374. doi:10.5966/sctm.2015-0326.

[348] R. Romero, J.K. Travers, E. Asbury, A. Pennybaker, L. Chubb, R. Rose, N.P. Ehrhart, M.J. Kipper, Combined delivery of FGF-2, TGF-β1, and adipose-derived stem cells from an engineered periosteum to a critical-sized mouse femur defect, J. Biomed. Mater. Res. Part A. 1 (2016) 1–39. doi:10.1002/jbm.a.35965.

[349] H.R.B. Raghavendran, S. Mohan, K. Genasan, M.R. Murali, S.V. Naveen, S. Talebian, R. McKean, T. Kamarul, Synergistic interaction of platelet derived growth factor (PDGF) with the surface of PLLA/Col/HA and PLLA/HA scaffolds produces rapid osteogenic differentiation, Colloids Surfaces B Biointerfaces. 139 (2016) 68–78. doi:10.1016/j.colsurfb.2015.11.053.

[350] T. Liu, X. Zhang, Y. Luo, Y. Huang, G. Wu, Slowly Delivered Icariin/Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells to Promote the Healing of Calvarial Critical-Size Bone Defects, Stem Cells Int. 2016 (2016). doi:10.1155/2016/1416047.

[351] R. Wadagaki, D. Mizuno, A. Yamawaki-Ogata, M. Satake, H. Kaneko, S. Hagiwara, N. Yamamoto, Y. Narita, H. Hibi, M. Ueda, Osteogenic induction of bone marrow-derived stromal cells on simvastatin-releasing, biodegradable, nano- to microscale fiber scaffolds, Ann. Biomed. Eng. 39 (2011) 1872–1881. doi:10.1007/s10439-011-0327-0.

[352] S. Peng, D. Gao, C. Gao, P. Wei, M. Niu, C. Shuai, MicroRNAs regulate signaling pathways in osteogenic differentiation of mesenchymal stem cells (Review), Mol. Med. Rep. 14 (2016) 623–629. doi:10.3892/mmr.2016.5335.

[353] D. Pezzoli, G. Candiani, Non-viral gene delivery strategies for gene therapy: A “ménage à trois” among nucleic acids, materials, and the biological environment: Stimuli-responsive gene delivery vectors, J. Nanoparticle Res. 15 (2013) 1523. doi:10.1007/s11051-013-1523-7.

[354] I. Mencía Castaño, C.M. Curtin, G.P. Duffy, F.J. O’Brien, Next generation bone tissue engineering: non-viral miR-133a inhibition using collagen-nanohydroxyapatite scaffolds rapidly enhances osteogenesis., Sci. Rep. 6 (2016) 27941. doi:10.1038/srep27941.

[355] T. Pan, W. Song, H. Gao, T. Li, X. Cao, S. Zhong, Y. Wang, MiR-29b-Loaded Gold Nanoparticles Targeting to the Endoplasmic Reticulum for Synergistic Promotion of Osteogenic Differentiation, ACS Appl. Mater. Interfaces. 8 (2016) 19217–19227. doi:10.1021/acsami.6b02969.

[356] X. Liu, B. Zeng, C. Zhang, Osteogenic and angiogenic effects of mesenchymal stromal cells with co-transfected human Ang-1 gene and BMP2 gene, Biotechnol. Lett. 33 (2011) 1933–1938. doi:10.1007/s10529-011-0654-0.

[357] H.-Y. Hsiao, S.-R. Yang, E.M. Brey, I.-M. Chu, M.-H. Cheng, Hydrogel Delivery of Mesenchymal Stem Cell–Expressing Bone Morphogenetic Protein-2 Enhances Bone Defect Repair, Plast. Reconstr. Surg. - Glob. Open. 4 (2016) e838. doi:10.1097/GOX.0000000000000817.

[358] H. Schneider, B. Sedaghati, A. Naumann, M.C. Hacker, M. Schulz-Siegmund, Gene Silencing of Chordin Improves BMP-2 Effects on Osteogenic Differentiation of Human Adipose Tissue-Derived Stromal Cells, Tissue Eng. Part A. 20 (2014) 335–345. doi:10.1089/ten.tea.2012.0563.

[359] I. Sandvig, I. Gadjanski, M. Vlaski, L. Buzanska, D. Loncaric, A. Sarnowska, L. Rodriguez, A. Sandvig, Z. Ivanovic, Strategies to enhance implantation and survival of stem cells after their injection in ischemic neural tissue, Stem Cells Dev. (2017) scd.2016.0268. doi:10.1089/scd.2016.0268.

[360] I. Rosová, M. Dao, B. Capoccia, D. Link, J. a. Nolta, Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells., Stem Cells. 26 (2008) 2173–2182. doi:10.1634/stemcells.2007-1104.

[361] A. Ravichandran, J. Lim, M.S.K. Chong, F. Wen, Y. Liu, Y.T. Pillay, J.K.Y. Chan, S.H. Teoh, In vitro cyclic compressive loads potentiate early osteogenic events in engineered bone tissue, J. Biomed. Mater. Res. - Part B Appl. Biomater. (2016) 1–10. doi:10.1002/jbm.b.33772.

[362] G.N. Bancroft, V.I. Sikavitsas, J. van den Dolder, T.L. Sheffield, C.G. Ambrose, J.A. Jansen, A.G. Mikos, Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12600–5. doi:10.1073/pnas.202296599.

[363] J. Filipowska, G.C. Reilly, A.M. Osyczka, A single short session of media perfusion induces

osteogenesis in hBMSCs cultured in porous scaffolds, dependent on cell differentiation stage, Biotechnol. Bioeng. 113 (2016) 1814–1824. doi:10.1002/bit.25937.

[364] G.M. de Peppo, G. Vunjak-Novakovic, D. Marolt, Cultivation of Human Bone-Like Tissue from Pluripotent Stem Cell-Derived Osteogenic Progenitors in Perfusion Bioreactors, Methods Mol. Biol. 1202 (2013) 173–184. doi:10.1007/7651_2013_52.

[365] A.B. Lovati, S. Lopa, C. Recordati, G. Talo, C. Turrisi, M. Bottagisio, M. Losa, E. Scanziani, M. Moretti, In Vivo Bone Formation Within Engineered Hydroxyapatite Scaffolds in a Sheep Model, Calcif. Tissue Int. 99 (2016) 209–223. doi:10.1007/s00223-016-0140-8.

[366] S. Rumiński, B. Ostrowska, J. Jaroszewicz, T. Skirecki, K. Włodarski, W. Święszkowski, M. Lewandowska-Szumieł, 3D printed PCL-based scaffolds provide an advantageous environment for osteogenic differentiation of human adipose-derived stem cells, J. Tissue Eng. Regen. Med. (2016) doi: 10.1002/term.2310. doi:10.1002/term.2310.

[367] G. Bilousova, D.H. Jun, K.B. King, S. De Langhe, W.S. Chick, E.C. Torchia, K.S. Chow, D.J. Klemm, D.R. Roop, S.M. Majka, Osteoblasts derived from induced pluripotent stem cells form calcified structures in scaffolds both in vitro and in vivo, Stem Cells. 29 (2011) 206–216. doi:10.1002/stem.566.

[368] B.Y.K. Binder, D.C. Genetos, J.K. Leach, Lysophosphatidic acid protects human mesenchymal stromal cells from differentiation-dependent vulnerability to apoptosis., Tissue Eng. Part A. 20 (2014) 1156–64. doi:10.1089/ten.TEA.2013.0487.

[369] K. von der Haar, A. Lavrentieva, F. Stahl, T. Scheper, C. Blume, Lost signature: progress and failures in in vivo tracking of implanted stem cells, Appl. Microbiol. Biotechnol. 99 (2015) 9907–9922. doi:10.1007/s00253-015-6965-7.

[370] A. Hagenhoff, C.J. Bruns, Y. Zhao, I. von Luttichau, H. Niess, C. Spitzweg, P.J. Nelson, Harnessing mesenchymal stem cell homing as an anticancer therapy, Expert Opin Biol Ther. 16 (2016) 1–14. doi:10.1080/14712598.2016.1196179.

[371] A. Klimczak, U. Kozlowska, Mesenchymal stromal cells and tissue-specific progenitor cells: Their role in tissue homeostasis, Stem Cells Int. 2016 (2016) 1–11. doi:10.1155/2016/4285215.

[372] K.E. Hatzistergos, D. Saur, B. Seidler, W. Balkan, M. Breton, K. Valasaki, L.M. Takeuchi, A.M. Landin, A. Khan, J.M. Hare, Stimulatory Effects of Mesenchymal Stem Cells on cKit+ Cardiac Stem Cells Are Mediated by SDF1/CXCR4 and SCF/cKit Signaling Pathways, Circ. Res. 119 (2016) 921–930. doi:10.1161/CIRCRESAHA.116.309281.

[373] N. Heldring, I. Mäger, M. Wood, K. Le Blanc, S. El Andaloussi, Therapeutic potential of multipotent mesenchymal stromal cells and their extracellular vesicles., Hum. Gene Ther. 26 (2015) 506–517. doi:10.1089/hum.2015.072.

[374] V. Neirinckx, G. Agirman, C. Coste, A. Marquet, V. Dion, B. Rogister, R. Franzen, S. Wislet, Adult bone marrow mesenchymal and neural crest stem cells are chemoattractive and accelerate motor recovery in a mouse model of spinal cord injury., Stem Cell Res. Ther. 6 (2015) 211. doi:10.1186/s13287-015-0202-2.

[375] Q. Wang, G. Ding, X. Xu, Immunomodulatory functions of mesenchymal stem cells and possible mechanisms, Histol. Histopathol. 31 (2016) 949–959. doi:10.14670/HH-11-750.

[376] X. Bai, Y. Yan, M. Coleman, G. Wu, B. Rabinovich, M. Seidensticker, E. Alt, Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging, Mol. Imaging Biol. 13 (2011) 633–645. doi:10.1007/s11307-010-0392-z.

[377] M. Vilalta, I.R. Dégano, J. Bagó, D. Gould, M. Santos, M. García-Arranz, R. Ayats, C. Fuster, Y. Chernajovsky, D. García-Olmo, N. Rubio, J. Blanco, Biodistribution, long-term survival, and safety of human adipose tissue-derived mesenchymal stem cells transplanted in nude mice by high sensitivity non-invasive bioluminescence imaging., Stem Cells Dev. 17 (2008) 993–1003. doi:10.1089/scd.2007.0201.

[378] S.R. Motamedian, S. Hosseinpour, M.G. Ahsaie, A. Khojasteh, Smart scaffolds in bone tissue engineering: A systematic review of literature., World J. Stem Cells. 7 (2015) 657–68. doi:10.4252/wjsc.v7.i3.657.

[379] G. Godeau, P. Barthélémy, Glycosyl-nucleoside lipids as low-molecular-weight gelators, Langmuir. 25 (2009) 8447–8450. doi:10.1021/la900140b.

[380] J.A. Kaplan, P. Barthélémy, M.W. Grinstaff, Self-assembled nanofiber hydrogels for mechanoresponsive therapeutic anti-TNFα antibody delivery, Chem. Commun. 52 (2016) 1–4. doi:10.1039/C6CC02221A.

[381] L. Latxague, M.A. Ramin, A. Appavoo, P. Berto, M. Maisani, C. Ehret, O. Chassande, P. Barthélémy, Control of Stem-Cell Behavior by Fine Tuning the Supramolecular Assemblies of Low-Molecular-Weight Gelators, Angew. Chemie - Int. Ed. 54 (2015) 4517–4521. doi:10.1002/anie.201409134.

[382] S. Meghezi, D.G. Seifu, N. Bono, L. Unsworth, K. Mequanint, D. Mantovani, Engineering 3D Cellularized Collagen Gels for Vascular Tissue Regeneration, J. Vis. Exp. (2015) e52812. doi:10.3791/52812.

[383] A. Ferreira, P. Gentile, V. Chiono, G. Ciardelli, Collagen for bone tissue regeneration, Acta Biomater. 8 (2012) 3191–3200.

[384] S. Mathews, R. Bhonde, P.K. Gupta, S. Totey, Extracellular matrix protein mediated regulation of the osteoblast differentiation of bone marrow derived human mesenchymal stem cells, Differentiation. 84 (2012) 185–192. doi:10.1016/j.diff.2012.05.001.

[385] B.A. Bunnell, M. Flaat, C. Gagliardi, B. Patel, C. Ripoll, Adipose-derived stem cells: Isolation, expansion and differentiation, Methods. 45 (2008) 115–120. doi:10.1016/j.ymeth.2008.03.006.

[386] A.L. Michaud, J.A. Herrick, J.E. Duplain, J.L. Manson, C. Hemann, P. Ilich, R.J. Donohoe, R. Hille, W.A. Oertling, FTIR characterization of heterocycles lumazine and violapterin in solution: effects of solvent on anionic forms, Biospectroscopy. 4 (1998) 235–256. doi:10.1002/(SICI)1520-6343(1998)4:4<235::AID-BSPY3>3.0.CO;2-1.

[387] C. Branco da Cunha, D.D. Klumpers, W.A. Li, S.T. Koshy, J.C. Weaver, O. Chaudhuri, P.L. Granja, D.J. Mooney, Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology, Biomaterials. 35 (2014) 8927–8936. doi:10.1016/j.biomaterials.2014.06.047.

[388] B.D. Walters, J.P. Stegemann, Strategies for directing the structure and function of three-dimensional collagen biomaterials across length scales, Acta Biomater. 10 (2014) 1488–1501. doi:10.1016/j.actbio.2013.08.038.

[389] L. Wang, J.P. Stegemann, Thermogelling chitosan and collagen composite hydrogels initiated with β-glycerophosphate for bone tissue engineering, Biomaterials. 31 (2010) 3976–3985. doi:10.1016/j.biomaterials.2010.01.131.

[390] M.B. Oliveira, C.A. Custodio, L. Gasperini, R.L. Reis, J.F. Mano, Autonomous osteogenic differentiation of hASCs encapsulated in methacrylated gellan-gum hydrogels, Acta Biomater. 41

(2016) 119–132. doi:10.1016/j.actbio.2016.05.033.

[391] U. Akalp, S.J. Bryant, F.J. Vernerey, Tuning tissue growth with scaffold degradation in enzyme-sensitive hydrogels: a mathematical model, Soft Matter. 12 (2016) 7505–7520. doi:10.1039/c6sm00583g.

[392] M. Mizuno, R. Fujisawa, Y. Kuboki, Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction, J. Cell. Physiol. 184 (2000) 207–213. doi:10.1002/1097-4652(200008)184:2<207::AID-JCP8>3.0.CO;2-U.

[393] L. Ma, C. Gao, Z. Mao, J. Zhou, J. Shen, X. Hu, C. Han, Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering, Biomaterials. 24 (2003) 4833–4841. doi:10.1016/S0142-9612(03)00374-0.

[394] S.K. Nandi, S. Roy, P. Mukherjee, B. Kundu, D.K. De, D. Basu, Orthopaedic applications of bone graft & graft substitutes: A review, Indian J. Med. Res. 132 (2010) 15–30. doi:10.1016/j.cuor.2005.12.001.

[395] K. Bergman, T. Engstrand, J. Hilborn, D. Ossipov, S. Piskounova, T. Bowden, Injectable cell-free template for bone-tissue formation, J. Biomed. Mater. Res. - Part A. 91 (2009) 1111–1118. doi:10.1002/jbm.a.32289.

[396] J. Hilborn, In vivo injectable gels for tissue repair, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 3 (2011) 589–606. doi:10.1002/wnan.91.

[397] P. Ducy, G. Karsenty, The family of bone morphogenetic proteins, Kidney Int. 57 (2000) 2207–2214. doi:10.1046/j.1523-1755.2000.00081.x.

[398] L. Zhang, Y. Ye, X. Long, P. Xiao, X. Ren, J. Yu, BMP signaling and its paradoxical effects in tumorigenesis and dissemination., Oncotarget. 7 (2016). doi:10.18632/oncotarget.12151.

[399] L. Kyllönen, M. D'Este, M. Alini, D. Eglin, Local drug delivery for enhancing fracture healing in osteoporotic bone, Acta Biomater. 11 (2015) 412–434. doi:10.1016/j.actbio.2014.09.006.

[400] C.H. Lee, M.U. Jin, H.M. Jung, J.T. Lee, T.G. Kwon, Effect of dual treatment with SDF-1 and BMP-2 on ectopic and orthotopic bone formation, PLoS One. 10 (2015). doi:10.1371/journal.pone.0120051.

[401] E. Argintar, S. Edwards, J. Delahay, Bone morphogenetic proteins in orthopaedic trauma surgery, Injury. 42 (2011) 730–734. doi:10.1016/j.injury.2010.11.016.

[402] J.N. Zara, R.K. Siu, X. Zhang, J. Shen, R. Ngo, M. Lee, W. Li, M. Chiang, J. Chung, J. Kwak, B.M. Wu, K. Ting, C. Soo, High Doses of Bone Morphogenetic Protein 2 Induce Structurally Abnormal Bone and Inflammation *In Vivo*, Tissue Eng. Part A. 17 (2011) 1389–1399. doi:10.1089/ten.tea.2010.0555.

[403] M. Papanagiotou, Z.H. Dailiana, T. Karachalios, S. Varitimidis, M. Hantes, G. Dimakopoulos, M. Vlychou, K.N. Malizos, Heterotopic ossification after the use of recombinant human bone morphogenetic protein-7, World J. Orthop. 8 (2017) 36. doi:10.5312/wjo.v8.i1.36.

[404] N. Sood, A. Bhardwaj, S. Mehta, A. Mehta, Stimuli-responsive hydrogels in drug delivery and tissue engineering., Drug Deliv. 7544 (2014) 1–23. doi:10.3109/10717544.2014.940091.

[405] D. Dyondi, T.J. Webster, R. Banerjee, A nanoparticulate injectable hydrogel as a tissue engineering scaffold for multiple growth factor delivery for bone regeneration, Int. J. Nanomedicine. 8 (2012) 47–59. doi:10.2147/IJN.S37953.

[406] F. Bastami, Z. Paknejad, M. Jafari, M. Salehi, M.R. Rad, A. Khojasteh, Fabrication of a three-dimensional β-tricalcium-phosphate/gelatin containing chitosan-based nanoparticles for sustained release of bone morphogenetic protein-2: Implication for bone tissue engineering, Mater. Sci. Eng. C. 72 (2016) 481–491. doi:10.1016/j.msec.2016.10.084.

[407] L. Cao, J.A. Werkmeister, J. Wang, V. Glattauer, K.M. McLean, C. Liu, Bone regeneration using photocrosslinked hydrogel incorporating rhBMP-2 loaded 2-N, 6-O-sulfated chitosan nanoparticles, Biomaterials. 35 (2014) 2730–2742. doi:10.1016/j.biomaterials.2013.12.028.

[408] R. Dong, Y. Pang, Y. Su, X. Zhu, Supramolecular hydrogels: synthesis, properties and their biomedical applications, Biomater. Sci. 3 (2015) 937–954. doi:10.1039/C4BM00448E.

[409] G.M. Peters, J.T. Davis, Supramolecular gels made from nucleobase, nucleoside and nucleotide analogs, Chem. Soc. Rev. 45 (2016) 3188–3206. doi:10.1039/C6CS00183A.

[410] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent., J. Biol. Chem. 193 (1951) 265–275. doi:10.1016/0304-3894(92)87011-4.

[411] H.E. Gruber, Adaptations of Goldner's Masson trichrome stain for the study of undecalcified plastic embedded bone., Biotech. Histochem. 67 (1992) 30–4. doi:10.3109/10520299209110002.

[412] E.J. Carragee, E.L. Hurwitz, B.K. Weiner, A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: Emerging safety concerns and lessons learned, Spine J. 11 (2011) 471–491. doi:10.1016/j.spinee.2011.04.023.

[413] E.J. Woo, Recombinant Human Bone Morphogenetic Protein 2, Yjoms. 70 (2012) 765–767. doi:10.1016/j.joms.2011.09.008.

[414] E.J. Woo, Adverse events reported after the use of recombinant human bone morphogenetic protein 2, J. Oral Maxillofac. Surg. 70 (2012) 765–767. doi:10.1016/j.joms.2011.09.008.

[415] E.J. Woo, Adverse events after recombinant human BMP2 in nonspinal orthopaedic procedures general, Clin. Orthop. Relat. Res. 471 (2013) 1707–1711. doi:10.1007/s11999-012-2684-x.

[416] A.K. Ekenseair, K.W.M. Boere, S.N. Tzouanas, T.N. Vo, F.K. Kasper, A.G. Mikos, Structure-property evaluation of thermally and chemically gelling injectable hydrogels for tissue engineering, Biomacromolecules. 13 (2012) 2821–2830. doi:10.1021/bm300797m.

[417] J. Louis-Ugbo, H.S. Kim, S.D. Boden, M.T. Mayr, R.C. Li, H. Seeherman, D. D'Augusta, C. Blake, A. Jiao, S. Peckham, Retention of 125I-labeled recombinant human bone morphogenetic protein-2 by biphasic calcium phosphate or a composite sponge in a rabbit posterolateral spine arthrodesis model, J. Orthop. Res. 20 (2002) 1050–1059. doi:10.1016/S0736-0266(02)00011-6.

[418] R.S. Sellers, R. Zhang, S.S. Glasson, H.D. Kim, D. Peluso, D.A. D'Augusta, K. Beckwith, E.A. Morris, Repair of articular cartilage defects one year after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2), J. Bone Jt. Surg. Am. Vol. 82 (2000) 151–160.

[419] M. Yamamoto, Y. Takahashi, Y. Tabata, Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenetic protein, Biomaterials. 24 (2003) 4375–4383. doi:10.1016/S0142-9612(03)00337-5.

[420] J.-Y. Jo, S.-I. Jeong, Y.-M. Shin, S.-S. Kang, S.-E. Kim, C.-M. Jeong, J.-B. Huh, Sequential delivery of BMP-2 and BMP-7 for bone regeneration using a heparinized collagen membrane, Int. J. Oral Maxillofac. Surg. 44 (2015) 921–928. doi:10.1016/j.ijom.2015.02.015.

[421] B.C. Jeong, H. Choi, S.W. Hur, J.W. Kim, S.H. Oh, H.S. Kim, S.C. Song, K.B. Lee, K.B. Park, J.T.

Koh, Repair of Cranial Bone Defects Using rhBMP2 and Submicron Particle of Biphasic Calcium Phosphate Ceramics with Through-Hole, Biomed Res. Int. 2015 (2015). doi:10.1155/2015/926291.

[422] H. Choi, B.C. Jeong, S.W. Hur, J.W. Kim, K.B. Lee, J.T. Koh, The angiopoietin-1 variant COMP-Ang1 enhances BMP2-induced bone regeneration with recruiting pericytes in critical sized calvarial defects, PLoS One. 10 (2015) 1–13. doi:10.1371/journal.pone.0140502.

[423] E. Quinlan, E.M. Thompson, A. Matsiko, F.J. O 'brien, A. López-Noriega, Long-term controlled delivery of rhBMP-2 from collagen–hydroxyapatite scaffolds for superior bone tissue regeneration, J. Control. Release. 207 (2015) 112–119. doi:10.1016/j.jconrel.2015.03.028.

[424] B.-R. Kim, T.B.L. Nguyen, Y.-K. Min, B.-T. Lee, In vitro and in vivo studies of BMP-2-loaded PCL-gelatin-BCP electrospun scaffolds., Tissue Eng. Part A. 20 (2014) 3279–89. doi:10.1089/ten.TEA.2014.0081.

[425] S.S. Kim, S.J. Gwak, B.S. Kim, Orthotopic bone formation by implantation of apatite-coated poly(lactide-co-glycolide)/hydroxyapatite composite particulates and bone morphogenetic protein-2, J. Biomed. Mater. Res. - Part A. 87 (2008) 245–253. doi:10.1002/jbm.a.31782.

[426] M. Rodríguez-Évora, A. Delgado, R. Reyes, A. Hernández-Daranas, I. Soriano, J. San Román, C. Évora, Osteogenic effect of local, long versus short term BMP-2 delivery from a novel SPUâ€"PLGAâ€"Î²TCP concentric system in a critical size defect in rats, Eur. J. Pharm. Sci. 49 (2013) 873–884. doi:10.1016/j.ejps.2013.06.008.

[427] P.D. Mariner, J.M. Wudel, D.E. Miller, E.E. Genova, S.-O. Streubel, K.S. Anseth, Synthetic hydrogel scaffold is an effective vehicle for delivery of INFUSE (rhBMP2) to critical-sized calvaria bone defects in rats, (n.d.). doi:10.1002/jor.22243.

[428] Y.-I. Chung, K.-M. Ahn, S.-H. Jeon, S.-Y. Lee, J.-H. Lee, G. Tae, Enhanced bone regeneration with BMP-2 loaded functional nanoparticle–hydrogel complex, J. Control. Release. 121 (2007) 91–99. doi:10.1016/j.jconrel.2007.05.029.

[429] V. Karageorgiou, M. Tomkins, R. Fajardo, L. Meinel, B. Snyder, K. Wade, J. Chen, G. Vunjak-Novakovic, D.L. Kaplan, Porous silk fibroin 3-D scaffolds for delivery of bone morphogenetic protein-2in vitro andin vivo, J. Biomed. Mater. Res. Part A. 78A (2006) 324–334. doi:10.1002/jbm.a.30728.

[430] S. Hyun, D. Han, S. Choi, J. Chai, K. Cho, Effect of Recombinant Human Bone, (2005) 1667–1674.

[431] S. Kamakura, S. Nakajo, O. Suzuki, Y. Sasano, New scaffold for recombinant human bone morphogenetic protein-2, J. Biomed. Mater. Res. - Part A. 71 (2004) 299–307. doi:10.1002/jbm.a.30157.

[432] D.H.R. Kempen, L. Lu, T.E. Hefferan, L.B. Creemers, A. Maran, K.L. Classic, W.J.A. Dhert, M.J. Yaszemski, Retention of in vitro and in vivo BMP-2 bioactivity in sustained delivery vehicles for bone tissue engineering, (n.d.). doi:10.1016/j.biomaterials.2008.04.031.

[433] Z. Huang, T.D. Sargeant, J.F. Hulvat, A. Mata, P. Bringas Jr., C.Y. Koh, S.I. Stupp, M.L. Snead, Bioactive nanofibers instruct cells to proliferate and differentiate during enamel regeneration, J. Bone Miner. Res. 23 (2008) 1995–2006. doi:10.1359/jbmr.080705.

[434] J. Lee, W. Il Choi, G. Tae, Y.H. Kim, S.S. Kang, S.E. Kim, S.-H. Kim, Y. Jung, S.H. Kim, Enhanced regeneration of the ligament–bone interface using a poly(L-lactide–co-e-caprolactone) scaffold with local delivery of cells/BMP-2 using a heparin-based hydrogel, Acta Biomater. 7 (2011) 244–257. doi:10.1016/j.actbio.2010.08.017.

[435] M.T. Poldervaart, H. Wang, J. van der Stok, H. Weinans, S.C.G. Leeuwenburgh, F.C. Öner, W.J.A. Dhert, J. Alblas, Sustained Release of BMP-2 in Bioprinted Alginate for Osteogenicity in Mice and Rats, PLoS One. 8 (2013) e72610. doi:10.1371/journal.pone.0072610.

[436] L. Gong, K. Hoshi, S. Ejiri, T. Nakajima, S. Shingaki, H. Ozawa, Bisphosphonate incadronate inhibits maturation of ectopic bone induced by recombinant human bone morphogenetic protein 2, J Bone Min. Metab. 21 (2003) 5–11.

[437] H. Maeda, A. Sano, K. Fujioka, Controlled release of rhBMP-2 from collagen minipellet and the relationship between release profile and ectopic bone formation, Int. J. Pharm. 275 (2004) 109–122. doi:10.1016/j.ijpharm.2004.01.040.

[438] C.S. Kim, J. Il Kim, J. Kim, S.H. Choi, J.K. Chai, C.K. Kim, K.S. Cho, Ectopic bone formation associated with recombinant human bone morphogenetic proteins-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers, Biomaterials. 26 (2005) 2501–2507. doi:10.1016/j.biomaterials.2004.07.015.

[439] E.J. Chung, K.B. Chien, B.A. Aguado, R.N. Shah, Osteogenic Potential of BMP-2-Releasing Self-Assembled Membranes, (n.d.). doi:10.1089/ten.tea.2012.0667.

[440] J. Ma, F. Yang, S.K. Both, H.-J. Prins, M.N. Helder, J. Pan, F.-Z. Cui, J.A. Jansen, J.J.J.P. van den Beucken, Bone forming capacity of cell- and growth factor-based constructs at different ectopic implantation sites, J. Biomed. Mater. Res. Part A. 103 (2015) 439–450. doi:10.1002/jbm.a.35192.

[441] H. Sun, J. Wang, F. Deng, Y. Liu, X. Zhuang, J. Xu, L. Li, Co-delivery and controlled release of stromal cell-derived factor-1α chemically conjugated on collagen scaffolds enhances bone morphogenetic protein-2-driven osteogenesis in rats, Mol. Med. Rep. 14 (2016) 737–745. doi:10.3892/mmr.2016.5339.

[442] M. Kisiel, A.S. Klar, M. Ventura, J. Buijs, M.K. Mafina, S.M. Cool, J. Hilborn, Complexation and Sequestration of BMP-2 from an ECM Mimetic Hyaluronan Gel for Improved Bone Formation, PLoS One. 8 (2013). doi:10.1371/journal.pone.0078551.

[443] B. Stevens, Y. Yang, A. Mohandas, B. Stucker, K.T. Nguyen, A review of materials, fabrication methods, and strategies used to enhance bone regeneration in engineered bone tissues, J. Biomed. Mater. Res. - Part B Appl. Biomater. 85 (2008) 573–582. doi:10.1002/jbm.b.30962.

[444] T.W. Bauer, G.F. Muschler, Bone Graft Materials, Clin. Orthop. Relat. Res. 371 (2000) 10–27. doi:10.1097/00003086-200002000-00003.

[445] A.C.L. Luna, M.E.P. Madeira, T.O. Conceição, J.A.L.C. Moreira, R.A.N. Laiso, D.A. Maria, Characterization of adipose-derived stem cells of anatomical region from mice., BMC Res. Notes. 7 (2014) 552. doi:10.1186/1756-0500-7-552.

[446] D.A. De Ugarte, K. Morizono, A. Elbarbary, Z. Alfonso, P.A. Zuk, M. Zhu, J.L. Dragoo, P. Ashjian, B. Thomas, P. Benhaim, I. Chen, J. Fraser, M.H. Hedrick, Comparison of multi-lineage cells from human adipose tissue and bone marrow., Cells. Tissues. Organs. 174 (2003) 101–9. doi:71150.

[447] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human Adipose Tissue Is a Source of Multipotent Stem Cells, Mol. Biol. Cell. 13 (2002) 4279–4295. doi:10.1091/mbc.E02-02-0105.

[448] S.J. Lee, N. Klar, J.C. Weeks, J.H. Antin, Predicting Costs of Stem-Cell Transplantation, J. Clin. Oncol. 18 (2000) 64–64. doi:10.1200/JCO.2000.18.1.64.

[449] C.A. Herberts, M.S. Kwa, H.P. Hermsen, P. Biglioli, M. Capogrossi, J. Nygren, J. Fries, K. Tiemann, H. Bohlen, J. Hescheler, M. Bhatia, Risk factors in the development of stem cell therapy, J. Transl. Med. 9 (2011) 29. doi:10.1186/1479-5876-9-29.

[450] Y. Li, S.K. Chen, L. Li, L. Qin, X.L. Wang, Y.X. Lai, Bone defect animal models for testing efficacy of bone substitute biomaterials, J. Orthop. Transl. 3 (2015) 95–104. doi:10.1016/j.jot.2015.05.002.

[451] J.O. Hollinger, J.C. Kleinschmidt, The critical size defect as an experimental model to test bone repair materials., J. Craniofac. Surg. 1 (1990) 60–8.

[452] J.O. Hollinger, Bone tissue engineering, CRC Press, 2005.

[453] J. Wang, M.J. Glimcher, Characterization of matrix-induced osteogenesis in rat calvarial bone defects: I. Differences in the cellular response to demineralized bone matrix implanted in calvarial defects and in subcutaneous sites., Calcif. Tissue Int. 65 (1999) 156–65.

Annexes 1 : Résultats complémentaires

Dans cette annexe, sont présentés des résultats qui ont été obtenus au cours de l'étude de la libération contrôlée de BMP-2 par les hydrogels de GNF. Bien que ces résultats n'aient pas été intégrés lors de la rédaction de l'article présenté au Chapitre 5 « Prolonged delivery of BMP-2 by a non-polymer hydrogel for bone defect regeneration », ils apportent des précisions complémentaires.

La micro-structure des hydrogels a été observée au cryo-microscope électronique à balayage à la plateforme de caractérisation des matériaux d'Aquitaine (PLACAMAT). Les images réalisées ont permis de mettre en évidence la structure des matériaux, composés de fibres. Ce réseau très dense permet la diffusion lente du facteur de croissance emprisonné au sein du réseau de fibres.

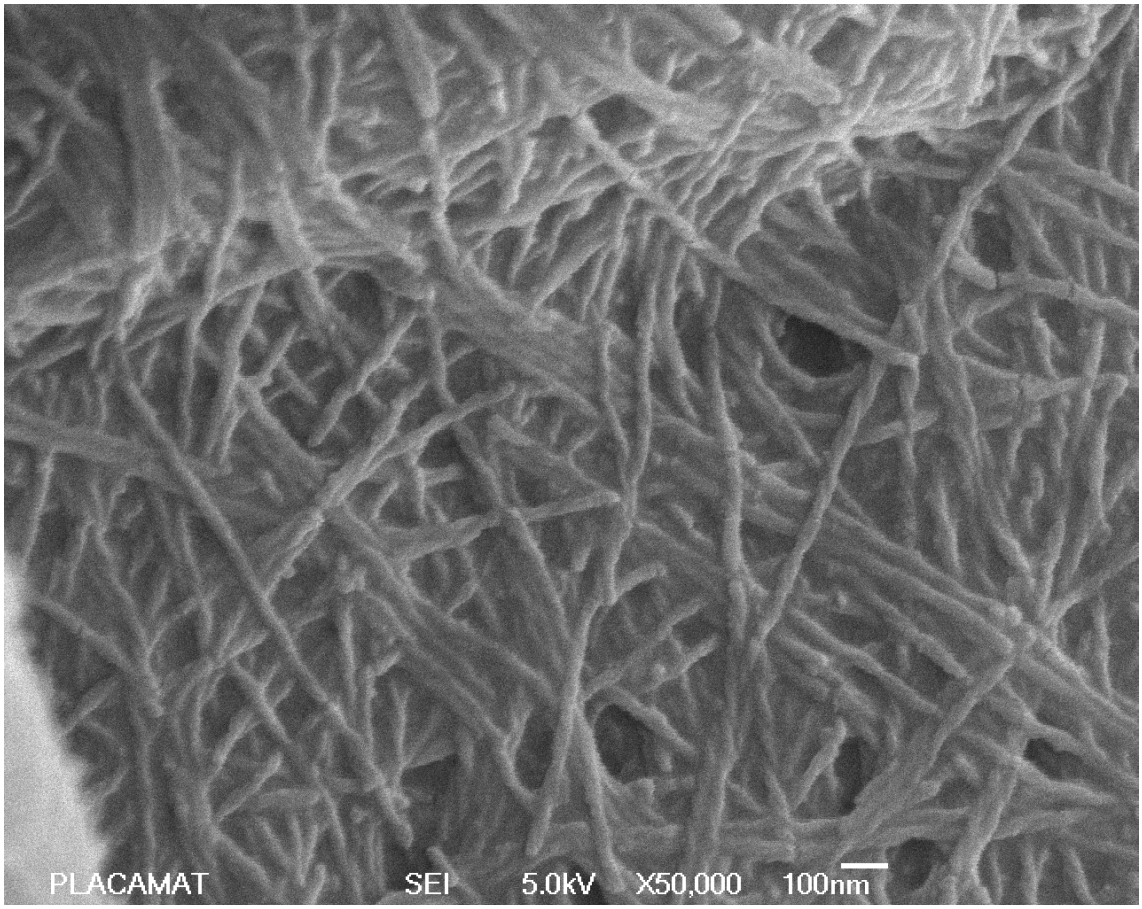

Figure 37 Observation en cryo-microscopie électronique à balayage

Dans un second temps, des injections en site sous-cutané d'hydrogels de GNF chargés en BMP-2 ont été réalisées chez la souris. Deux mois après la chirurgie les animaux ont été sacrifiés et les hydrogels injectés ont pu être prélevés. L'observation des coupes histologiques de ces prélèvements montrait l'infiltration des blocs de GNF par des cellules ostéo-formatrices. De plus, des dépôts de tissus minéralisés (colorés en violet) étaient observés sur la surface des gels de GNF (colorés en bleu).

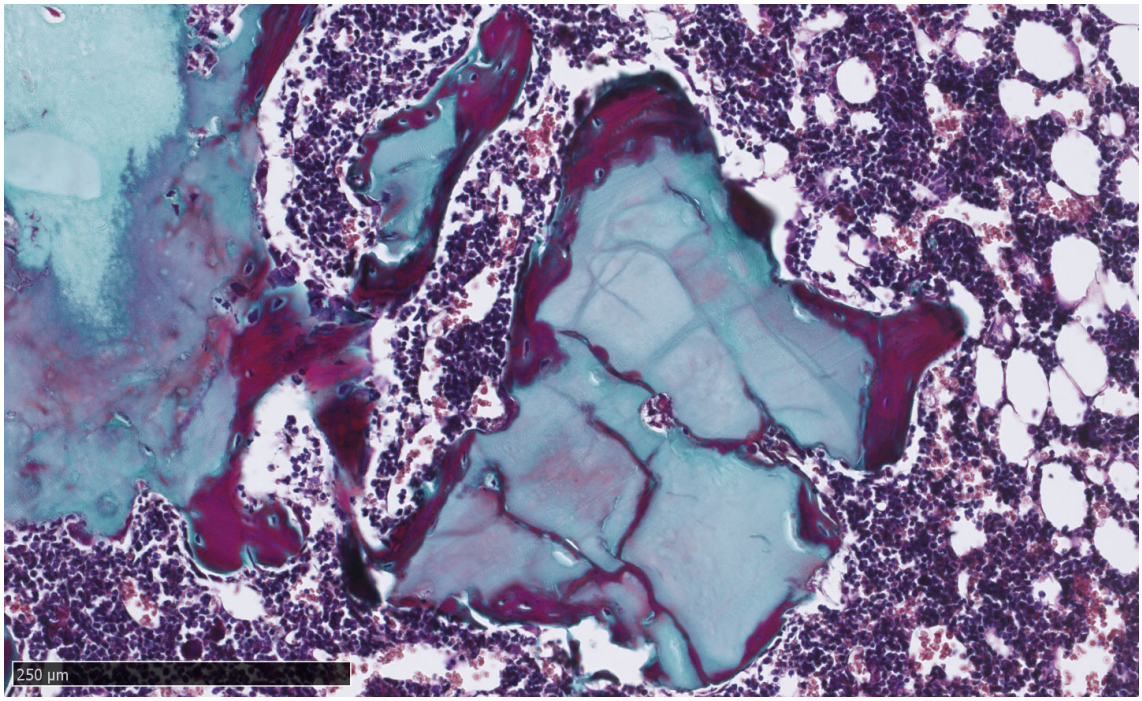

Figure 38 Mineralisation et infiltration de cellules de l'hôte au sein de l'hydrogel

Des injections des hydrogels de GNF chargés en BMP-2 ont également été réalisées sur des lésions de calvaria comme présenté dans le chapitre 5. Les résultats présentés au chapitre 5 concerne les données quantitatives de régénération et de qualité osseuse. Est présenté en annexe une image représentative de la reconstruction tridimensionnelle acquises par imagerie micro-scanner. Ces analyses ont été réalisées au sein de l'unité de recherche EA2496 (Laboratoires Pathologies, Imagerie et Biothérapies orofaciales, Université Paris Descartes).

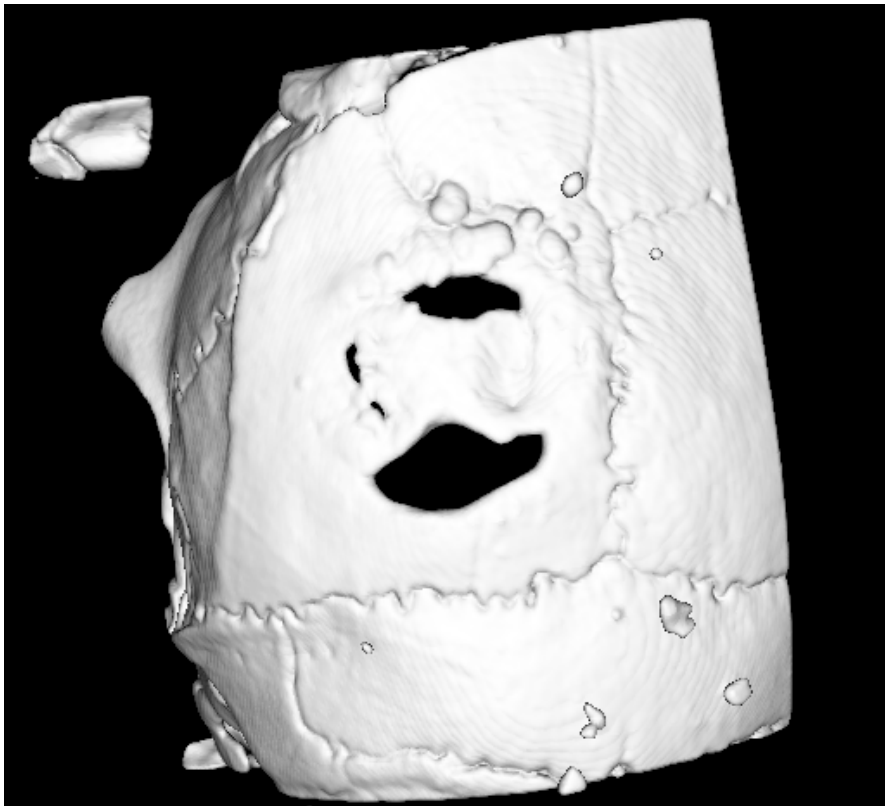

Figure 39 Reconstruction tridimensionnelle par technique d'imagerie µCT des explants de calvaria

Annexes 2 : Communications scientifiques

Publications dans des journaux scientifiques (5)

• Control of Stem-Cell Behavior by Fine Tuning the Supramolecular Assemblies of Low-Molecular- Weight Gelators

L. Latxague, M. Ramin, A. Appavoo, P. Berto, **M. Maisani**, C. Ehret, O. Chassande, and P. Barthélémy

Angewandte Chemie, Publié, Février 2015, (IF: 11.709)

• Design, development and validation of a new composite hydrogel for bone tissue engineering

M.Maisani, R. Bareille, L. Levesque, J. Amédée, D. Mantovani, O. Chassande

Bone Joint Journal, Publié, Janvier 2017 (compte rendu de congrès)

• A new composite hydrogel combining the biological properties of collagen with the mechanical properties of a supramolecular scaffold for bone tissue engineering

M. Maisani, C. Ehret, L. Levesque, R. Siadous, J.-F. Le Meins, P. Chevalier, P. Barthélémy, H. Oliveira, J. Amédée, D. Mantovani, O. Chassande

Journal of tissue engineering and regenerative medicine, En révision, Décembre 2016, (IF: 3.989)

• Soft scaffolds to repair hard tissues: How to create a physiologically-relevant microenvironment?

M. Maisani, D.Pezzoli, O.Chassande, D. Mantovani

Journal of Tissue Engineering, Publié, Avril 2017

• Hydrogel as BMP-2 reservoir and delivery system for bone regeneration

M. Maisani, S. KR, M. Fénelon, R. Siadous, J-C. Fricain, D. Mantovani, O.Chassande

Drug delivery and translational research, En révision, Juin 2017 (IF: 3.094)

Communications internationales (7)

Communications orales (3)

• European Orthopaedic Research Society, 2016, Bologne, Italie

Design, development and validation of a new composite hydrogel for bone tissue engineering

M. Maisani, R. Bareille, L. Levesque, J. Amédée, D. Mantovani, O. Chassande

• Canadian Biomaterial Society, 2015, Toronto, Ontario, Canada

Hybrid hydrogel as a microenvironment for tissue engineering: A new matrix for bone regeneration.

M. Maisani, L. Levesque, R. Bareille, O. Chassande, D. Mantovani

• Canadian Biomaterials Society, 2014, Halifax, Nova Scotia, Canada

Glycosyl-Nucleosyl-Fluorinated – Collagen injectable hydrogel for tissue engineering: a new scaffold for bone regeneration

M. Maisani, L. Levesque, C. Ehret, R. Bareille, O. Chassande, D. Mantovani

Communications affichées (4)

• 1er Forum Franco-Québécois d'innovation en Santé, 2016, Montréal, Canada

Design, development and validation of a new composite hydrogel for bone tissue engineering

M. Maisani, R. Siadous, C. Ehret ; R. Bareille, L. Levesque, P. Barthélémy, J. Amédée, D. Mantovani, O. Chassande

• 10th World Biomaterials Congress, 2016, Montréal, Canada

A new hydrogel microfiber for cell and growth factor delivery designed bone tissue engineering

M. Maisani, J. Kalisky, R. Bareille, L. Levesque, O. Chassande, D. Mantovani

• 10th World Biomaterials Congress, 2016, Montréal, Canada

Hybrid hydrogel for tissue engineering: A new matrix for bone regeneration

M. Maisani, L. Levesque, R. Bareille, O. Chassande, D. Mantovani

• Canadian Biomaterial Society, 2015, Toronto, Ontario, Canada

A Layer-by-Layer Biofabrication for Tissue Engineering

V. Guduric, C. Metz, **M. Maisani**, R. Siadous, R. Levato, R. Bareille, E. Engel, J.-C. Fricain, O. Luzanin, S. Catros

Communications nationales (8)

Communications orales (5)

• 2nd Workshop on Regenerative Medicine, 2016, Bordeaux

Design, development and validation of a new composite hydrogel for bone tissue engineering

M. Maisani, R. Bareille, L. Levesque, J. Amédée, D. Mantovani, O. Chassande

• Congrès BIOMAT, 2015, Ile de Ré

Design, development and validation of a new composite hydrogel for bone tissue engineering

M. Maisani, R. Bareille, L. Levesque, J. Amédée, D. Mantovani, O. Chassande

• Journée de la FR Tecsan, Institut Européen de Chimie et Biologie, 2015, Pessac

Hybrid hydrogel as a microenvironment for tissue engineering: A new matrix for bone regeneration.

M. Maisani, L. Levesque, R. Bareille, J. Amédée, O. Chassande, D. Mantovani

• 17ème Journées Françaises de Biologie des Tissus Minéralisés, 2015, Clermont-Ferrand

Glycosyl-Nucleosyl-Fluorinated – Collagen injectable hydrogel for tissue engineering: a new scaffold for bone regeneration

M. Maisani, C. Ehret, R. Bareille, L. Levesque, P. Barthélemy, J. Amédée, D. Mantovani, O. Chassande

• 4ème Journées du GDR GRIMIT 2014, Lyon

Hydrogel de Collagène-GNF injectable pour l'ingénierie tissulaire : un échafaudage prometteur pour la régénération osseuse

M. Maisani, C. Ehret, R. Bareille, L. Levesque, P. Barthélemy, J. Amédée, D. Mantovani, O. Chassande

Communications affichées (3)

• 18ème Journées Françaises des Biomatériaux et Tissus Minéralisés 2015, Nancy

Hybrid hydrogel for tissue engineering: A new matrix for bone regeneration

M. Maisani, C. Ehret, L. Levesque, R. Bareille, O. Chassande, D. Mantovani

• 17ème Journées Françaises des Biomatériaux et Tissus Minéralisés 2015, Clermont-Ferrand

Glycosyl-Nucleosyl-Fluorinated – Collagen injectable hydrogel for tissue engineering: a new scaffold for bone regeneration

M. Maisani, C. Ehret, L. Levesque, R. Bareille, Diego Mantovani, O. Chassande, J. Amédée

• 16ème Journées Françaises des Biomatériaux et Tissus Minéralisés, Limoges 2014

Glycosyl-Nucleosyl-Fluorinated – Collagen injectable hydrogel for tissue engineering: a new scaffold for bone regeneration

M. Maisani, C. Ehret, L. Levesque, R. Bareille, Diego Mantovani, O. Chassande, J. Amédée