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INFLUENCE DE FILMS DE POLYCAPROLACTONE FONCTIONNALISÉS PAR DES PEPTIDES D'ADHÉSION SUR LA SIGNALISATION INTRACELLULAIRE DE CELLULES SOUCHES : RÉGULATION DE LA RÉPONSE AUX FACTEURS DE CROISSANCE

Mémoire de maîtrise
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Le talent est un cadeau, mais le succès ne vient qu'avec le travail
Jean Béliveau

RÉSUMÉ

Le vieillissement de la population augmentera l'incidence des troubles osseux, notamment les fractures ostéoporotiques, menant ainsi à des pertes osseuses de taille critique. La méthode privilégiée afin de combler ces pertes, l'autogreffe, comporte cependant des limitations. L'une des alternatives étudiées est l'utilisation de matériaux biomimétiques. Afin d'optimiser la régénération osseuse, les biomatériaux doivent interagir avec le tissu environnant. Ainsi, ces matériaux peuvent être fonctionnalisés avec des peptides d'adhésion, le peptide Arg-Gly-Asp (RGD) étant l'une des séquences les plus fréquemment utilisées. De plus, afin de favoriser la différenciation des cellules en ostéoblastes, l'utilisation de facteurs de croissance tels que les protéines morphogénétiques osseuses (BMPs) s'avère efficace, la BMP-2 et la BMP-7 étant approuvées par la *Food and Drug Administration* pour des applications commerciales de régénération osseuse. La BMP-9 a montré avoir un potentiel ostéogénique supérieur à ces dernières. Par contre, peu d'études portent sur l'influence des peptides d'adhésion sur la réponse des cellules aux BMPs. Ce projet de maîtrise a donc pour principal objectif d'étudier l'impact d'un film de polycaprolactone (PCL) fonctionnalisé par des peptides d'adhésion dérivés de la sialoprotéine osseuse (PCL-pBSP) ou de la fibronectine (PCL-pFibro) sur la réponse de cellules souches mésenchymateuses (MSCs) C3H10T1/2 à la BMP-9 et à ses peptides dérivés (pBMP-9 et SpBMP-9). En effet, les BMPs étant coûteux à produire et purifier, des peptides dérivés moins dispendieux ont été développés.

Le premier volet de ce mémoire consiste en une revue de la littérature qui vient d'être acceptée dans le journal *Frontiers in Bioscience*. Le tissu osseux, le processus de réparation osseuse et les types d'interactions entre les cellules osseuses et les biomatériaux y sont décrits. Elle présente aussi le processus de différenciation ostéogénique des MSCs afin d'identifier les molécules pouvant être utilisées dans le développement de matériaux biomimétiques en plus de décrire les plus récents matériaux biomimétiques fonctionnalisés par des peptides d'adhésion utilisés conjointement avec des facteurs de croissance.

Le deuxième volet décrit les résultats expérimentaux obtenus qui font l'objet d'un article soumis dans le journal *Acta Biomaterialia*. Il a été démontré que le PCL-pFibro par rapport au PCL-pBSP permettait une meilleure organisation du cytosquelette des C3H10T1/2 et une activation plus rapide de la *focal adhesion kinase*, protéine impliquée dans l'adhésion cellulaire. De plus, les 2 films de PCL fonctionnalisés ont montré un effet contraire au niveau de la signalisation JNK; le PCL-pFibro l'activait tandis que le PCL-pBSP l'inhibait. Or JNK est indispensable à la différenciation ostéogénique des C3H10T1/2 induite par la BMP-9. Suite à une stimulation des MSCs par la BMP-9 ou ses peptides dérivés, les C3H10T1/2 adhérant sur le PCL-pFibro ont montré une activation et une translocation nucléaire des Smad1/5/8 plus importantes que sur PCL-pBSP. À plus long terme, l'expression de Runx2, marqueur de la différenciation ostéogénique des MSCs, était plus importante sur PCL-pFibro que sur PCL-pBSP.

En conclusion, ces travaux ont permis de démontrer le potentiel du PCL-pFibro comme matériau biomimétique capable d'induire une signalisation intracellulaire favorable à l'action de la BMP-9 et de ses peptides dérivés dans un processus de différenciation ostéogénique des MSCs. Ce matériau pourrait donc être prometteur dans une stratégie de réparation des pertes osseuses en combinaison avec le pBMP-9 ou SpBMP-9.

Mots-clés : biomatériaux, facteurs de croissance, cellules osseuses, peptides d'adhésion, signalisation cellulaire

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ACS	*Absorbable collagen type I sponge*
AdBMP	*Adenovirus encoding BMP*
AdSC	*Adipose derived stem cell*
ALP	Phosphatase alcaline, *alkaline phosphatase*
ANOVA	Analyse de variance, *analysis of variance*
AP-1	*Activating protein-1*
APC	*Adenomatous polyposis coli*
ASARM	*Acidic serine-rich and aspartate-rich motif*
BAMBI	*BMP and activin membrane-bound inhibitor*
BCP	*Biphasic calcium phosphate*
Beta T	*Beta tail*
BMP	Protéine morphogénétique osseuse, *bone morphogenetic protein*
BMPR	Récepteur des BMPs, *BMP receptor*
BSA	Albumine bovine sérique, *bovine serum albumin*
BSP	Sialoprotéine osseuse, *bone sialoprotein*
CAS	*Crk-associated substrate*
CBD	*Decapeptide collagen-binding*
Cdc42	*Cell division cycle 42*
CPTES	*(3-chloropropyl) triethoxysilane*
CSL	*CBF1, Suppressor of Hairless, Lag-1*
DAG	*Diacylglycerol*
DAP12	*12 kDa DNAX-activating protein*
DAPI	4-6-diamidino-2-phénylindole
DC-STAMP	*Dendritic cell-specific transmembrane protein*
DGEA	Peptide Asp-Gly-Glu-Ala
Dkk-1	Dickkopf-1
Dvl	Dishevelled
E1	*Epidermal growth factor domain 1*
ECM	Matrice extracellulaire
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EGF	*Epidermal growth factor*
EphB4	*Ephrin type-B receptor 4*
ERK1/2	*Extracellular signal regulated kinase 1/2*
F-actin	Actine filamenteuse, *filamentous actin*
FAK	*Focal adhesion kinase*
FBS	Sérum bovin foetal, *foetal bovine serum*
FDA	*Food and Drug Administration*
FERM	*N-terminal ezrin radixin moesin*
FGF	*Fibroblast growth factor*

FGFR	*FGF receptor*
Fzd	Frizzled
GAP	*GTPase activating protein*
GEF	*Guanine exchange factor*
GFs	*Growth factors*
Grb2	*Growth factor receptor-bound protein 2*
GSK-3 beta	*Glycogen synthase kinase-3 beta*
HAP	Hydroxyapatite
Hes	*Hairy enhancer of split*
Hey	*Hes-related with the YRPW motif*
Hh	Hedgehog
HMEC-1	*Microvascular endothelial-1 cells*
hMSC	MSC humaine, *human MSC*
IFN-gamma	*Interferon-gamma*
IGF	*Insulin-like growth factor*
IGFIR	*IGF type I receptor*
IL	*Interleukin*
ILK	*Integrin-linked kinase*
IP3	Inositol-1,4,5-trisphosphate
IRS-1	*Insulin receptor substrate-1*
I-Smad	*Inhibitory Smad*
JNK	c-Jun N-terminal kinase
KRSR	Lys–Arg–Ser–Arg
LRP5/6	*Low-density lipoprotein receptor-related protein 5 and 6*
MAML	Mastermind-like
MAPK	*Mitogen-activated protein kinase*
M-CSF	*Macrophage-colony stimulating factor*
MEPE	*Matrix extracellular phosphoglycoprotein*
MMP	*Matrix metalloproteinases*
MSC	Cellule souche mésenchymateuse, *mesenchymal stem cell*
NFATc1	*Nuclear factor of activated T cells cytoplasmic 1*
NHS	N-hydroxysuccinimide
NICD	*Notch intracellular domain*
OC	Ostéocalcine, *osteocalcin*
OPG	Ostéoprotégérine, *osteoprotegerin*
OPN	Ostéopontine, *osteopontin*
OSCAR	*Osteoclast-associated receptors*
Osx	Ostérix, *osterix*
pBMP	Peptide dérivé des BMPs, *BMP derived peptide*
pBMP-2	Peptide dérivé de la BMP-2, *peptide derived from BMP-2*
pBMP-9	Peptide dérivé de la BMP-9, *peptide derived from BMP-9*

PBS	Tampon phosphate salin, *phosphate-buffered saline*
pBSP	Peptide RGD issu de la sialoprotéine osseuse
PCL	Polycaprolactone
PCL-Hydro	PCL hydrolysé
PCL-pBSP	PCL fonctionnalisé par un peptide issu de la sialoprotéine osseuse
PCL-pFibro	PCL fonctionnalisé par un peptide issu de la fibronectine
PDEA	2-(2-pyridinyldithio) ethaneamine hydrochloride
PDGF	*Platelet-derived growth factor*
PEG	*Poly (ethylene) glycol*
pERK1/2	ERK1/2 phosphorylé, *phosphorylated ERK1/2*
PET	*Polyethylene terephthalate*
PI3K	*Phosphatidyl inositol 3 kinase*
pFAK	FAK phosphorylée, *phosphorylated FAK*
pFibro	Peptide issu de la fibronectine
PGA	Acide polyglycolique
pJNK	JNK phosphorylé, *phosphorylated JNK*
PLA	Poly(lactide)
PLCgamma	*Phospholipase C gamma*
PLEOF	*Poly(lactide-ethylene oxide fumarate)*
PLGA	Poly(DL-lactide-co-glycolide)
PLLA	Poly (L-lactide)
p-p38	p38 phosphorylé, *phosphorylated p38*
PPARγ	*Peroxisome proliferator-activated receptor gamma*
PPM1A	*Protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A*
PS	Polystyrène
pSmad1/5/8	Smad1/5/8 phosphorylé, *phosphorylated Smad1/5/8*
PTH	*Parathyroid hormone*
Rac1	*Ras-related C3 botulinum toxin substrate 1*
RANKL	*Receptor activator of NF-kappaB ligand*
RGD	Tripeptide Arg-Gly-Asp
Runx2	*Runt-related transcription factor 2*
SEM	*Standard errors of the mean*
Sema4D	Semaphorin 4D
Shc	*Src homology 2 domain-containing*
SIBLING	*Small integrin-binding ligand N-linked glycoproteins*
siRNA	*Small interfering RNA*
SLRP	*Small leucine-rich proteoglycans*
Smad1/5/8	*Small mothers against decapentaplegic1/5/8*
Sos	*Son of sevenless*
Sox9	*Sex determining region Y-box 9*
SP-1	Sphingosine-1 phosphate

STKR	*Ser/Thr kinase receptor*
TAK1	*TGF-β1-activated tyrosine kinase 1*
TCF/LEF	*T-cell factor/lymphoid enhancer factor*
TGF-β	Facteurs de croissance transformant bêta
Ti	Titane, *titanium*
TiO$_2$	Dioxyde de titane, *titanium dioxide*
TNF	*Tumor necrosis factor*
TRAF6	*TNF receptor associated factor-6*
TRAP	*Tartrate resistant acid phosphatase*
TREM-2	*Triggering receptor expressed in myeloid cells 2*
VEGF	*Vascular endothelial growth factor*
WASP	*Wiscott-Aldrich syndrome protein*
WHO	*World Health Organization*
XIAP	*X-linked inhibitor of apoptosis*

CHAPITRE 1. INTRODUCTION

1.1 Mise en contexte

Selon Statistique Canada, le nombre de personnes âgées de plus de 65 ans passera de 4,9 millions en 2011 à 10,9 millions en 2036, représentant ainsi 25% de la population [279,280]. Ce vieillissement entraînera une augmentation du nombre de troubles osseux, notamment des fractures ostéoporotiques, menant à des fractures de taille critique. Selon Ostéoporose Canada, plus de 80% des fractures subies par les personnes de plus de 50 ans sont dues à l'ostéoporose [215]. Cela aura aussi un effet au niveau économique. Ainsi, il en coûte au Canada 1,9 milliard de dollars annuellement afin de traiter l'ostéoporose et les fractures associées et ces coûts devraient se chiffrer à 32,5 milliards de dollars en 2018 [214].

Afin de combler les pertes osseuses engendrées par des fractures, chiffrées à plus de 188 000 en 2007 au Canada [217], l'une des techniques utilisées et qui est considérée comme étant le standard en or par les chirurgiens orthopédistes est l'autogreffe [86,232]. Elle consiste à prélever le greffon à même le patient, plus particulièrement au niveau de la crête iliaque [86,232]. L'autogreffe possède la propriété de pouvoir être colonisée par des cellules osseuses (ostéoconduction), induire la différenciation des cellules souches mésenchymateuses (MSCs) en ostéoblastes matures (ostéoinduction) et être capable d'interagir et de s'intégrer au niveau du site receveur (ostéointégration) [86]. L'autogreffe comporte cependant certaines limitations notamment au niveau de la taille du greffon pouvant être prélevée ainsi que de la morbidité et de la douleur induite au niveau du site donneur [86,232]. De plus, le greffon doit posséder des propriétés mécaniques similaires à celles de l'os où il sera implanté. Les greffons provenant de la crête iliaque sont principalement composés d'os spongieux et ne fournissent que très peu de support mécanique [232]. Une autre technique utilisée est l'allogreffe, qui consiste à prélever le greffon sur un cadavre d'un individu de la même espèce. L'allogreffe permet d'avoir une plus grande quantité de tissu osseux disponible et évite la morbidité au site donneur [194]. Par contre, il y a risque de rejet du greffon dû à une réponse immunologique ainsi qu'une faible possibilité de transmission de maladies telles que le virus de l'immunodéficience humaine (1 cas sur 1,6 million) [27,265,274].

Afin de pallier ces problèmes, de nouvelles stratégies ont donc été développées, notamment l'utilisation de biomatériaux. Ces matériaux doivent posséder les mêmes caractéristiques que l'autogreffe, soit l'ostéoconduction, l'ostéoinduction et l'ostéointégration. Les matériaux utilisés comme substituts osseux se regroupent en 4 catégories, soit les matériaux inorganiques, les polymères naturels et synthétiques ainsi que les matériaux composites [160,180]. Tous ces matériaux sont ostéoconductifs et ostéointégratifs. Par contre, ils sont peu ou pas ostéoinductifs [180]. Parmi les matériaux inorganiques, pouvant être résorbables ou non, se trouvent entre autres l'hydroxyapatite, le tricalcium phosphate, les ciments de phosphate de calcium et le sulfate de calcium [180]. Les polymères naturels tels que le collagène, la fibrine et le chitosane sont extraits d'animaux ou de plantes [83,156,169,212]. Ceux-ci sont biodégradables, mais ont le désavantage de pouvoir transmettre des agents pathogènes [160,169]. Les polymères synthétiques comprennent notamment le poly(lactide) (PLA), le poly(glycolide) (PGA) et leurs copolymères (poly(DL-lactide-co-glycolide (PLGA)) ainsi que le polycaprolactone (PCL) [160,226]. Ceux-ci possèdent le désavantage de produire des produits de dégradation acides pouvant nuire à la réparation osseuse. Quant aux matériaux composites, ils sont formés d'un mélange de matériaux inorganiques et de polymères synthétiques ou naturels afin de tirer avantage des caractéristiques de chacun des matériaux [160].

Le PCL représente un matériau d'intérêt pour la fabrication de substituts osseux. Celui-ci possède un temps de dégradation supérieur à d'autres matériaux utilisés en régénération osseuse (PCL : supérieur à 24 mois, le PGA : 6 à 12 mois et PLA : 12 à 16 mois) [185,251]. Cette résorption plus lente limite la réponse inflammatoire et permet une meilleure reconstruction osseuse [251]. De plus, le PCL est actuellement utilisé dans des substituts osseux approuvés par la *Food and Drug Administration* (FDA), soit Osteoplug[TM] et Osteomesh[TM] [213].

Cependant, le PCL ne permet pas une bonne interaction avec les cellules. Il doit donc être fonctionnalisé soit par des protéines d'adhésion de la matrice extracellulaire (ECM) soit par des peptides dérivés de ces protéines afin de favoriser l'attachement des cellules [57,103,334]. En effet, les cellules interagissent avec certaines séquences spécifiques des protéines de l'ECM via les intégrines, des récepteurs transmembranaires composés d'une

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sous-unité α et d'une sous-unité β pouvant former un total de 24 hétérodimères αβ [118]. L'une des séquences peptidiques les plus fréquemment retrouvées au sein de protéines telles que la fibronectine, la sialoprotéine osseuse et la vitronectine est le tripeptide Arg-Gly-Asp (RGD) [224]. De nombreux peptides comportant la séquence RGD ont donc été utilisés pour fonctionnaliser le PCL [13,57]. Par exemple, Drevelle *et al.* a utilisé un peptide issu de la sialoprotéine osseuse contenant la séquence RGD (CGGNGEPRGDTYRAY, pBSP) [57]. Le pBSP, contrairement au peptide négatif RGE (CGGNGEPRGETYRAY), a permis l'étalement des préostéoblastes murins MC3T3-E1 ainsi que la formation de points focaux d'adhésion, l'organisation du cytosquelette d'actine et la phosphorylation de la *focal adhesion kinase* (FAK). De plus, seules les cellules incubées sur le pBSP ont permis une activation de la voie des *small mothers against decapentaplegic1/5/8 (*Smad1/5/8) suite à une stimulation par la protéine morphogénétique osseuse-2 (BMP-2).

Les BMPs, faisant partie de la famille des facteurs de croissance transformant bêta (TGF-β), tout comme les cytokines pro-inflammatoires et les facteurs angiogéniques sont les cytokines régulant le processus de réparation osseuse. Parmi les facteurs angiogéniques se trouvent entre autres les *vascular endothelial growth factors* (VEGFs) et l'angiopoïétine qui permettent le développement d'une vascularisation nécessaire à une bonne réparation osseuse [3]. Lors du processus de régénération osseuse, plusieurs BMPs interviennent, notamment la BMP-2 présente à toutes les étapes de réparation [3]. Il existe 20 BMPs organisés en sous-familles en fonction de leur homologie de séquence [18]. Les BMPs se fixent à des récepteurs transmembranaires sérine/thréonine kinase de type I et II via leurs épitopes *wrist* (type I) et *knuckle* (type II) [140,191]. Cette interaction des BMPs avec les cellules permet d'activer la voie des Smad et la voie des *mitogen-activated protein kinase* (MAPKs), permettant à leur tour d'activer la transcription de gènes codant pour des marqueurs ostéogéniques [261]. Il a été démontré que les BMP-2/-4/-6/-7/-9 pouvaient induire la différenciation des MSCs murines C3H10T1/2 en ostéoblastes [40]. À l'heure actuelle, la FDA n'autorise que la BMP-2 et la BMP-7 pour des applications de régénération osseuse au niveau commercial [18,28,313]. Certaines études ont cependant démontré que la BMP-9 aurait un pouvoir ostéogénique supérieur à celui de la BMP-2 ou la BMP-7, tant au niveau *in vitro* qu'*in vivo* [40,132].

Puisque les BMPs ont un coût de production et de purification élevé, des peptides dérivés des BMPs (pBMP) ont été développés [38,247]. L'équipe de recherche du Prof. Nathalie Faucheux (Chaire de Recherche du Canada de niveau 2 : Systèmes biohybrides cellules-biomatériaux) a développé un peptide dérivé de la BMP-9 (pBMP-9). Celui-ci correspond à l'épitope *knuckle* de la BMP-9, soit la séquence reconnue par le récepteur de type II. Le pBMP-9 est 300 fois moins coûteux que la BMP-9 et aurait un effet similaire à celle-ci sur les préostéoblastes murins [16]. De plus, le pBMP-9 (100 µg) dans un système de libération à base de chitosane s'est montré être efficace pour induire un début de minéralisation dans le quadriceps de souris après 24 jours [14].

Cependant, l'utilisation de matériaux biomimétiques sur la réponse des cellules aux BMPs et à leurs peptides dérivés est peu étudiée. Il a été démontré que RGD, qui cible les intégrines $\alpha_v\beta_3$, permet une augmentation de l'activité de la phosphatase alcaline (ALP) chez les préostéoblastes murins MC3T3-E1 stimulés par pBMP-9, contrairement au peptide Asp-Gly-Glu-Ala (DGEA) qui cible les intégrines $\alpha_2\beta_1$ [179]. Par ailleurs, il a été démontré que la fibronectine, qui cible les intégrines $\alpha_v\beta_3$ et $\alpha_5\beta_1$, permet une augmentation de la réponse de cellules endothéliales à la BMP-9 [294].

L'utilisation de biomatériaux fonctionnalisés en combinaison avec les pBMPs semble donc une avenue prometteuse afin d'optimiser la réparation osseuse, malgré le peu d'études sur ce sujet [98,179,342].

1.2 Définition du projet de recherche

À la suite de la problématique présentée ci-dessus, il a été possible de poser l'hypothèse suivante : un matériau biomimétique de PCL fonctionnalisé par des peptides d'adhésion peut contrôler la réponse des MSCs C3H10T1/2 à la BMP-9 et à ses peptides dérivés.

Les peptides d'adhésion choisis pour ce projet sont le peptide RGD issu de la sialoprotéine osseuse (pBSP) et un peptide issu de la fibronectine (pFibro) qui cible les intégrines $\alpha_5\beta_1$ [134]. Puisque les cellules osseuses expriment principalement les intégrines $\alpha_5\beta_1$ et en plus faible proportion les intégrines $\alpha_v\beta_3$ [7], le pFibro devrait favoriser l'adhésion cellulaire ainsi que l'activation des voies de signalisation par la BMP-9 et ses peptides dérivés.

Par ailleurs, les peptides dérivés de la BMP-9 qui sont utilisés sont le pBMP-9 et le SpBMP-9. La particularité du SpBMP-9 est que 2 cystéines ont été remplacées par 2 sérines dans sa séquence d'acides aminés. Les travaux de Saito *et al.* ont permis de démontrer que ce changement au niveau d'un peptide dérivé de la BMP-2 permet une meilleure affinité du peptide pour le récepteur de type II, se traduisant par une activité plus importante du marqueur ostéogénique phosphatase alcaline (ALP) chez les MSCs C3H10T1/2 après 3 jours (100 µg de peptide dérivé de la BMP-2) [247]. Le SpBMP-9 devrait, par analogie avec l'étude sur la BMP-2, résulter en une meilleure activation des voies de signalisation ainsi qu'une meilleure orientation vers la lignée ostéoblastique des MSCs.

1.3 Objectifs de recherche

Afin de valider ou d'infirmer l'hypothèse, le projet a été divisé en 3 objectifs principaux, chacun étant divisé en sous-objectifs (Tableau 1.1).

Tableau 1.1 Objectifs généraux et spécifiques

Objectifs généraux	Objectifs spécifiques
1. Vérifier l'influence du pFibro et du pBSP sur l'adhésion des MSCs en préparant des films de PCL fonctionnalisés	1.1 Mettre en évidence la présence des intégrines par immunobuvardage de type Western et l'organisation des points focaux d'adhésion par immunomarquage et observation en microscopie à fluorescence 1.2 Déterminer l'activation de la FAK par immunobuvardage de type Western et immunomarquage 1.3 Déterminer l'activation des MAPK (ERK, p38, JNK)
2. Déterminer l'influence des films de PCL fonctionnalisés sur la réponse des cellules C3H10T1/2 attachées à ceux-ci en présence de BMP-9 ou ses peptides dérivés en termes de transduction du signal	2.1 Analyser l'activation de voies de signalisation (Smad et MAPK) par une concentration équimolaire de BMP-9, pBMP-9 ou SpBMP-9 2.2 Comparer la translocation des Smad phosphorylés au niveau du noyau
3. Déterminer la capacité des cellules C3H10T1/2 attachées aux films de PCL fonctionnalisés à se différencier en présence de BMP-9 ou ses peptides dérivés	3.1 Analyser la réponse cellulaire précoce par la mesure de l'expression de marqueurs ostéogéniques précoces (*Runt-related transcription factor 2*; Runx2) 3.2 Vérifier l'absence de chondrogenèse et d'adipogenèse par la mesure de l'expression génique de marqueurs adipocytaires (*Peroxisome proliferator-activated receptor gamma*; PPARγ) et chondrogéniques (*Sex determining region Y-box 9*; Sox9) 3.3 Vérifier si la différenciation ostéogénique induite par pBMP-9 et SpBMP-9 est JNK dépendante

1.4 Contributions originales

1.4.1 État de l'art

Le chapitre 2 présente une revue de littérature sur les interactions entre les cellules osseuses et les biomatériaux. Il s'agit, suite à l'invitation de l'éditeur en chef du journal, d'une mise à jour de l'article de revue *Bone cells-biomaterials interactions* publié par Marquis *et al.* dans la revue Fr*ontiers in Bioscience* [180]. Cet article de revue apporte les contributions suivantes :

- Une description du tissu osseux et des cellules qui le composent ainsi que le rôle de ces cellules sont présentés.
- La régulation du remodelage osseux et de la différenciation ostéogénique est décrite.
- Les adhésions focales et fibrillaires impliquées dans l'adhésion des cellules à des biomatériaux sont décrites ainsi que les nouvelles avancées au niveau de la constitution de l'adhésome.
- La synergie entre les intégrines ciblées par différentes protéines de l'ECM et les facteurs de croissance est présentée.
- Les plus récents travaux sur l'impact des matériaux biomimétiques fonctionnalisés par des peptides d'adhésion utilisés en réparation osseuse sur la réponse cellulaire en combinaison ou non avec des facteurs de croissance sont décrits et discutés.

1.4.2 Effet du PCL fonctionnalisé sur la réponse de cellules souches mésenchymateuses en présence de peptides dérivés de la BMP-9

Le chapitre 3 a permis de démontrer l'impact de cibler les intégrines $\alpha_5\beta_1$ par un peptide issu de la fibronectine par rapport au peptide RGD qui cible les intégrines $\alpha_v\beta_3$ en termes d'adhésion, d'activation de voies de signalisation et de différenciation chez les MSCs C3H10T1/2 en présence de peptides dérivés de la BMP-9. Les résultats ont permis la soumission d'un article dans la revue *Acta Biomaterialia*. Cet article a permis les contributions suivantes :

- Le PCL fonctionnalisé par pFibro (PCL-pFibro) permet une meilleure organisation des points focaux et du cytosquelette d'actine des MSCs par rapport au PCL fonctionnalisé par pBSP (PCL-pBSP).

- Le PCL-pFibro permet d'obtenir une phosphorylation de la FAK (Y397 et Y576/577) plus rapide chez les MSCs par rapport au PCL-pBSP.
- Le PCL-pFibro induit chez les MSCs une augmentation de la phosphorylation de c-Jun N-terminal kinase (JNK) alors qu'une diminution de la phosphorylation de JNK est observée chez les cellules adhérant au PCL-pBSP.
- Le PCL-pFibro permet une activation de la voie des Smad1/5/8 plus rapide et plus intense chez les MSCs stimulées par BMP-9, pBMP-9 ou SpBMP-9 comparativement aux cellules adhérant au PCL-pBSP.
- Le PCL-pFibro permet une meilleure translocation des Smad1/5 au noyau chez les MSCs stimulées par la BMP-9, pBMP-9 ou SpBMP-9 comparativement aux cellules adhérant au PCL-pBSP.
- La stimulation des MSCs par BMP-9, pBMP-9 ou SpBMP-9 permet de maintenir le niveau de phosphorylation de JNK retrouvé en l'absence de facteur de croissance chez les cellules adhérant au PCL-pFibro. Chez les cellules adhérant au PCL-pBSP, l'ajout de BMP-9 ou de ses peptides dérivés permet d'induire la phosphorylation de JNK.
- Le PCL-pFibro permet une meilleure expression de Runx2 chez les MSCs C3H10T1/2 que le PCL-pBSP. L'ajout d'un inhibiteur de JNK diminue l'expression de Runx2 chez les MSCs adhérant au PCL-pFibro et au PCL-pBSP en présence de BMP-9, pBMP-9 ou SpBMP-9.

1.5 Plan du document

Le mémoire de maîtrise comporte 4 chapitres. Le premier chapitre présente la problématique reliée aux problèmes squelettiques ainsi que différentes stratégies pour combler les pertes osseuses. Ce chapitre comprend aussi l'hypothèse de recherche, les objectifs y étant associés ainsi que les contributions originales. Le chapitre 2 correspond à l'état de l'art sur les interactions entre les cellules osseuses et les biomatériaux sous la forme d'une revue de littérature acceptée dans *Frontiers in Bioscience*. Le chapitre 3 présente les résultats du projet sous la forme d'un article scientifique soumis dans *Acta Biomaterialia* portant sur l'impact du peptide d'adhésion pFibro ou pBSP sur la réponse des MSCs murins C3H10T1/2 en présence de BMP-9 ou de peptides dérivés au niveau de l'adhésion cellulaire et de l'activation de voies

de signalisation. La conclusion du mémoire ainsi que les perspectives de recherche sont présentées au chapitre 4.

CHAPITRE 2.   ÉTAT DE L'ART

Titre original : Interactions between bone cells and biomaterials: An update

Titre français : Interactions entre les cellules osseuses et les biomatériaux : Une mise à jour

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Contributions au document :

Cet article contribue au mémoire en présentant les éléments intervenant dans ce projet. Les aspects suivants sont abordés:

- Le tissu osseux et les cellules osseuses.
- Le processus du remodelage osseux et de la différenciation ostéogénique ainsi que les différents facteurs les régulant.
- Les types d'adhésions impliquées dans l'interaction de cellules osseuses avec des biomatériaux ainsi que les réactions croisées entre les intégrines impliquées et les facteurs de croissance.
- Une revue des plus récents matériaux biomimétiques fonctionnalisés par des peptides d'adhésion utilisés en réparation osseuse et leur influence sur la réponse des cellules en présence de facteurs de croissance.

2.1 Résumé français

La population occidentale étant vieillissante, les gens souffriront davantage de défauts osseux dus à l'ostéoporose (non-union de fractures, dommages vertébraux), aux cancers (ostéolyse maligne) et aux infections (ostéomyélite). Les autogreffes sont généralement utilisées pour combler ces défauts, mais elles comportent plusieurs désavantages, notamment la morbidité au site donneur ainsi que la quantité et la qualité de greffon osseux pouvant être prélevé. De récents progrès scientifiques effectués dans le développement des biomatériaux se sont montrés prometteurs afin de surmonter ces limitations. Les interactions des cellules avec les biomatériaux peuvent être améliorées par l'ajout à leur surface de groupes fonctionnels comme des peptides d'adhésion et/ou des facteurs de croissance. Le développement de ces matériaux biomimétiques capables de contrôler la réponse des cellules osseuses ne peut se faire que s'il est basé sur une compréhension du comportement des cellules osseuses et leur régulation. Cette revue se concentre sur la physiologie de l'os ainsi que la régulation de la différenciation des cellules osseuses et leurs fonctions et comment les dernières avancées dans les matériaux biomimétiques peuvent être traduites en des résultats cliniques prometteurs.

2.2 Abstract

As the populations of the Western world become older, they will suffer more and more from bone defects related to osteoporosis (non-union fractures, vertebral damages), cancers (malignant osteolysis) and infections (osteomyelitis). Autografts are usually used to fill these defects, but they have several drawbacks such as morbidity at the donor site and the amount and quality of bone that can be harvested. Recent scientific milestones made in biomaterials development were shown to be promising to overcome these limitations. Cell interactions with biomaterials can be improved by adding at their surface functional groups such as adhesive peptides and/or growth factors. The development of such biomimetic materials able to control bone cell responses can only proceed if it is based on a sound understanding of bone cell behavior and regulation. This review focuses on bone physiology and the regulation of bone cell differentiation and function, and how the latest advances in biomimetic materials can be translated within promising clinical outcomes.

2.3 Introduction

People over 65 years old make up the most rapidly growing Canadian population [229]. Five million Canadians were over 65 years old in 2011, and this number is estimated to double by 2036, so that they will account for a quarter of the population [279,280]. The World Health Organization (WHO) estimates that the worldwide population aged over 60 will reach 2 billion by 2050 [316]. These older people are most at risk of bone disorders like secondary metastasis causing bone cancer or osteoporotic fractures which are leading causes of pain and disability [315]. Osteoporosis Canada reports that over 80 percent of the fractures sustained by people over 50 years old are caused by osteoporosis [216]. Major bone loss associated with osteoporosis (particularly in vertebral fractures or non-union fractures) can be difficult to repair, so becoming a significant financial burden. The annual cost to Canadians of treating osteoporosis and the resulting fractures could reach 30 billion dollars by 2018 [214].

Bone is the basic support of locomotion system, where muscles, ligaments and tendons are attached to it. It also provides mechanical support and protects vital organs. It contains bone marrow, the main site of hematopoiesis and an important reservoir of minerals [180,205]. Osteointegrative, osteoconductive and osteoinductive biocompatible bone substitutes should, ideally, be used to heal fractures and bridge bone losses [180]. Osteointegration requires extensive interaction between the bone substitute and the recipient's bone site.

Osteoconduction can only occur if the bone substitute is readily colonized by bone cells and blood vessels, while osteoinductive substitute must be able to stimulate host's mesenchymal stem cells (MSCs) from surrounding tissues to differentiate into bone-forming cells [86].

Biological graft such as autografts, the gold standard used by surgeons, and allografts are the most commonly used [86]. Autografts use patient's own living bone tissue, while allografts use cadaveric bone. Autografts provide an osteoconductive scaffold, osteogenic cells and osteoinductive growth factors, while allografts have a limited range of these properties [86]. However, the size of an autograft is limited by the amount of bone that can be harvested, and 8.5 to 20 percent of cases suffer from postoperative complications like nerve injury, infections, blood loss, morbidity and chronic pain at the donor and/or recipient's sites [56,86]. Most grafts are harvested from iliac crests so as to limit structural modification, but their size may vary considerably (5 to 70 cm^2) [25,47]. Larger grafts can be taken from the tibia or femur, providing generally greater quantities of osteogenic growth factors such as bone morphogenetic proteins (BMPs) (13). These donor sites do not provide horseshoe shaped grafts consisting of cortical bone (tricortical graft) that give greater structural support and mechanical resistance, unlike the iliac crest. In contrast to autograft bone, allograft is frozen in liquid nitrogen and undergoes a serie of treatments such as purification (NaHCO$_3$, H$_2$O$_2$, NaOH), conditioning and sterilization. These treatments have a considerable effect on the biological and mechanical properties of bone tissue [63,75,76]. With modern procurement and sterilization methods for bone tissue, the risk of infection transmission by allografts such as human immunodeficiency virus is estimated to be 1 in 2.8 billion [90,246].

Biomaterials with the same characteristics as bone grafts have to be developed to overcome these problems (limited graft size, morbidity and chronic pain) [102]. The biomaterial may be inorganic, like hydroxyapatite (HAP) or titanium (Ti), natural, such as collagen or alginate, or synthetic polymer like polycaprolactone (PCL), polylactate, or even a composite material [180]. Inorganic biomaterials like calcium phosphate ceramics have the same physical properties as bone mineral and induce a minimal immune response *in vivo* during implantation. However, the solubility of the calcium phosphate ceramics influences the activity of osteoclasts, the cells responsible for bone resorption [102,192]. Other materials, including synthetic polymers, can be broken down over time and replaced by regenerated

tissue in the long term [102]. However, they interact poorly with bone cells, resulting in the development of third generation biomaterials, such as biomimetic materials [58,158,180].

Several strategies have been used to create biomimetic materials. One of them is to functionalize the biomaterial by adding extracellular matrix (ECM) proteins, recombinant growth factors and/or peptides derived from them in order to mimic bone as close as possible to its physiology [4,18,98,99,154,228]. The therapeutic potential of these biomimetic materials depends on their capacity to control the behavior of MSCs [200]. Biomimetic materials must first promote the adhesion of MSCs to their surface and favor the response of these cells to specific growth factors leading to their differentiation into bone-forming cells. The interactions between cell membrane receptor integrins and the proteins/peptides at the surface of the biomimetic material play a crucial role in this phenomenon [30,234].

In the present review, we first describe the principal components of bone and their roles in bone healing and remodeling. We then look at the adhesion of bone cells to ECM and biomaterials and the crucial role of cell-biomaterial interactions in the integration and repair of bone tissue. Finally, the roles of each of these elements will be set in the context of the latest advances in the field of biomimetic materials.

2.4 Bone cells and the extracellular matrix

2.4.1 Bone cells

Bone remodeling is a physiological process in which bone resorption is followed by the formation of new bone. The cells responsible for these interrelated processes include the bone-resorbing cells, i.e. osteoclasts, which are derived from hematopoietic cells of the monocyte-macrophage lineage, and bone-forming cells, i.e. osteoblasts, which differentiate from bone marrow MSCs. Osteoblasts, osteoblast-derived osteocytes and osteoclasts are highly specialized bone cells [20].

Mature osteoblasts have a lifespan of about 3 months, and are protein-secreting cells with a well-developed rough endoplasmic reticulum and a large Golgi apparatus [177]. They synthesize the collagen-based matrix, the osteoid, at a rate of 2 to 3 microns^3 per day and mineralize it 10 days after its deposition. As a result, osteoblasts become surrounded by mineralized tissue. Osteoblasts that cover the bone surface become inactive lining cells, or

they die by apoptosis. Osteoblasts can control the differentiation of bone resorbing osteoclasts by secreting the receptor activator of NF-kappaB ligand (RANKL) and osteoprotegerin (OPG) [29].

Osteocytes make up 90 to 95 percent of all bone cells and have a half-life of about 25 years [142]. They originate from osteoblasts once they have become enclosed within the mineralized tissue. They are interconnected together via adherent and gap junctions. The osteocytes are sensors of bone mechanical stimuli and the microdamage induced by cyclic loading. They may also respond to fluid flow induced by strain in the canaliculi [311]. Osteocytes secrete major bone-regulating factors. They are the main source of RANKL in skeletal tissue, and also produce OPG and sclerostin, which influence the activity of other specialized bone cells [196]. Bone microdamages may also cause osteocytes to enter apoptosis, which favors the release of chemotactic signals that target osteoclasts [306].

Osteoclasts are large multinucleated cells with diameter about 50 to 100 microns formed by the fusion of monocytes, which are mononuclear cells. Osteoclasts have a lifespan of about 2 weeks. They become activated when they attached to the bone matrix [125,177]. Osteoclasts are highly motile and alternate between migratory and bone-resorbing stages, showing remarkable changes in their phenotype during these phases. When adhered to the bone, the osteoclasts become polarized and reorganize their cytoskeleton. A sealing zone is formed by densely packed actin-rich podosomes that delimit the ruffled border, a highly specialized area composed of membrane expansions directed toward the targeted bone surface. The ruffled border is formed by polarized vesicular trafficking and plays a critical role in the degradation of bone matrix through acidification by vacuolar H^+ ATPases and degrading enzymes released by the fusion of secretory lysosomal vesicles such as matrix metalloproteinases (MMP) and cathepsin K, or acid phosphatases such as tartrate resistant acid phosphatase (TRAP). These proteases can degrade the mineralized osteoid to form Howship lacunae 40 to 60 microns deep [303]. Osteoclasts also transport vesicles from basal to the apical cell membrane by transcytosis that contain calcium and phosphate ions and hydrolyzed osteoid proteins released during bone resorption [204]. In non-resorptive or migrating osteoclasts, the sealing zone switches to a podosome belt, and relaxed osteoclasts are depolarized [211].

2.4.2 Osteoid and mineral phase

Osteoid accounts for 20 to 25 percent of the bone mass. It is made up of over 90 percent of type I collagen [85,210]. Collagen fibrils (15 to 500 nm in diameter) are stabilized by intramolecular and intermolecular crosslinks formed by covalent, electrostatic and hydrogen bonds [93]. Collagen fibrils can co-assemble to form collagen fibers of about 10 microns in diameter [93]. The structure and the organization of collagens influence the mechanical properties of bone, such as its ductility and fracture resistance [81].

Osteoid matrix also contains around 5 percent of non-collagenous proteins. These are proteins like the small integrin-binding ligand N-linked glycoproteins (SIBLING), such as osteopontin (OPN), matrix extracellular phosphoglycoprotein (MEPE) and bone sialoprotein (BSP) [71]. SIBLING proteins undergo extensive post-translational modifications, including N/O- linked glycosylation, sulfation and/or phosphorylation, that influence their function (for review see [278]). They are important in the regulation of bone cell function and matrix mineralization, acting via their RGD sites and their acidic serine-rich and aspartate-rich motif (ASARM) [1,116,152,278]. Holm *et al.* recently found that the trabecular bones of BSP$^{-/-}$ knockout mice were less well mineralized than those of their wild type controls [108].

Adhesive proteins like fibronectin are also crucial for bone: they interact with cells via integrins to regulate their activity [207,259]. Schwab *et al.* recently reported that fibronectin was better than vitronectin for the adherence of human bone marrow MSCs and their differentiation into osteoblasts [259].

Osteoid also contains the small leucine-rich proteoglycans (SLRPs), biglycan and decorin, whose central protein cores are linked by glycosaminoglycans such as chondroitin sulfate [120]. Ingram *et al.* showed that biglycan was present in both cortical and trabecular bone, while decorin was mainly located in the canaliculi of osteocytes and in the matrix near the Haversian canals [120]. The collagen fibrils are abnormal in decorin$^{-/-}$ and biglycan$^{-/-}$ double-knockout mice [48]. SLRPs can regulate the hydrostatic and osmotic pressures as well as the transport of nutrients and growth factors [17]. Chen *et al.* used neonatal murine calvarial cells extracted from biglycan$^{-/-}$ knockout mice and adenovirus encoding biglycan to show that this proteoglycan was required for the osteogenic differentiation of calvarial cells induced by

BMP-4 [37]. SLRPs also play a key role in regulating the mechanical properties of bone, especially its poroelasticity [17].

The mineral part of bone is almost entirely made of HAP crystals, constituted of calcium and phosphate ions. It accounts roughly for 65 percent of the bone mass [70]. It also contains other ions - fluoride, manganese and magnesium - with some carbonate substitution. Mineralization of the osteoid begins with the nucleation of calcium phosphate followed by crystal growth [305]. Nucleation can develop from supersaturated concentrations of calcium and phosphate ions. It occurs in specific vesicles that bud off from the membranes of hypertrophic chondrocytes and osteoblasts and in the interstitial space through the action of specific SIBLING proteins [305]. The apatite crystals first form in the gaps between the collagen molecules. Several recent *in vitro* studies have suggested that collagen itself has specific sites rich in charged amino acid that favor crystal nucleation [43,206,320]. Wang *et al.* found that collagen could sequester enough calcium, phosphate and carbonate ions to favor their spontaneous transformation into apatite crystals [310]. However, there is still some debate about the results of these studies because most of them used tendon or isolated collagen fibrils as models, which lack the SIBLING proteins.

2.5 Bone cell differentiation and bone healing

2.5.1 Signaling pathways contributing to osteogenic differentiation of mesenchymal stem cells

The use of MSCs in tissue engineering is a promising strategy to enhance bone healing and regeneration [91]. However, fundamental understanding of the osteoblastic commitment capacity of implanted MSCs and its regulation will be critical [91]. The differentiation of MSCs into osteoblasts, chondrocytes, and adipocytes is regulated by growth factors, cytokines, hormones and vitamins (Figure 2.1) [143].
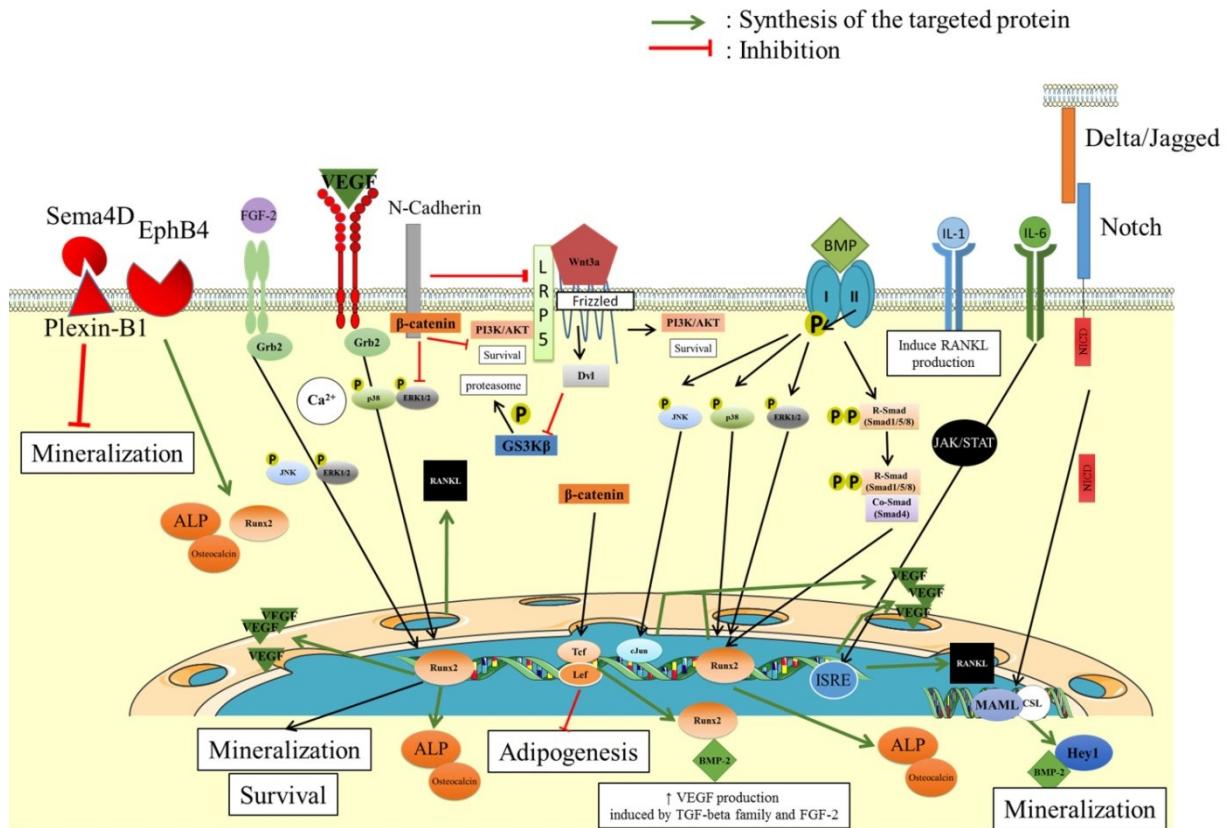
Figure 2.1 Signaling pathways regulating the differentiation of MSCs into osteoblasts (ALP, alkaline phosphatase; CSL, CBF1 Suppressor of Hairless Lag-1; EphB4, Ephrin type-B receptor 4; Hey, Hes-related with the YRPW motif; MAML, Mastermind-like; NICD, Notch intracellular domain; PI3K, phosphatidyl inositol 3 kinase; Sema4D, semaphorin 4D; VEGF, vascular endothelial growth factor [2,5,295,336,32,44,45,97,159,202,203,281]. [Illustration using Servier Medical Art, http://www.servier.fr]

Many growth factors, including fibroblast growth factor (FGF), insulin-like growth factor (IGF), the transforming growth factor-beta family (TGF-beta) and the platelet-derived growth factor (PDGF), are involved in the differentiation of MSCs into osteoblasts [9,40,115,174]. For example, Baker *et al.* found that bone ossification was abnormally slow in IGF-1$^{-/-}$ mice [9].

FGF and IGF bind to receptors belonging to the tyrosine kinase receptor family, FGF receptor (FGFR) for FGF and IGF type I receptor (IGFIR) for IGF. The receptors bearing their growth factors form dimers that are activated by trans-phosphorylation of their tyrosine residues. These receptors then recruit intracellular adaptor proteins such as growth factor receptor-bound protein 2 (Grb2) and (Src homology 2 domain-containing)-transforming

protein (Shc). The son of sevenless (Sos) is then recruited and the extracellular signal regulated kinase 1/2 (ERK1/2) mitogen activated protein kinase (MAPK) cascade is activated. This cascade can trigger the differentiation of MSCs into osteoblasts [188,270].

The BMPs also play a crucial part in bone tissue formation. More than 20 BMPs have been identified to date [18,200]. These molecules are synthesized by the MSCs and osteoblasts and are members of the TGF-beta family [178,286]. Marshall Urist showed that implanting demineralized bone in a muscle led to *de novo* bone formation. He also discovered that BMPs gave the organic bone matrix its osteoinductive properties [300]. Each kg of demineralized bone matrix contains 1 – 2 microg BMPs [171,233,317]. The osteogenic potential of BMPs has been verified *in vivo* by injecting C2C12 cells transformed with adenovirus encoding BMPs (AdBMP) into mouse quadriceps muscles [132]. Kang *et al.* found that AdBMP-6 or AdBMP-9 triggered ossification most rapidly and efficiently, followed by AdBMP-2 and AdBMP-7[132]. Like other members of the TGF-beta family, BMPs act on cells by inducing two type I and two type II serine/threonine kinase receptors to form a heterotetrameric complex. A total of 7 type I receptors and 5 type II receptors have been identified to date. They can bind over 30 TGF-beta family ligands and all have similar structures [137]. For example, the kinase domains of the BMP type I receptors (BMPR) BMPR-IA and BMPR-IB share 85 percent amino acids homology [137].

There are two pathways involved in BMP signaling, the canonical Smad pathway and a pathway involving TGF-beta activated tyrosine kinase 1 (TAK1) and MAPK [271]. In the canonical Smad pathway, after BMPs binding to receptors, the type I receptor is phosphorylated by the type II receptor, which in turn leads to the phosphorylation of Smad1/5/8 [190]. The phosphorylated Smad1/5/8 then form a complex with Smad4. This complex is translocated to the nucleus, where it activates the transcription of osteogenic genes like Runx2, osterix (Osx) and osteocalcin (OC) [182,190,309]. Liu *et al.* reported that small interfering RNA (siRNA) against Smad1 reduced the amount of ALP mRNA induced by BMP-2 in MC3T3-E1 preosteoblasts and inhibited matrix mineralization [165].

The canonical Smad pathway is regulated at many levels (Figure 2.2). The number of available BMP receptors at the cell surface can be modulated by endocytosis. Extracellular regulation occurs when antagonists such as Noggin, Chordin and Gremlin bind to BMPs and

20

inhibit their interaction with their receptors [21,223,341]. The Smad pathway is also regulated by the transmembrane pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI), which interacts with BMPRI to prevent the transduction of the signal [209]. The inhibitory Smads (I-Smad), Smad6 and Smad7, are intracellular regulators of the Smad pathway. They bind to the intracellular domain of type I receptors to form a stable complex that prevents the activation of Smad1/5/8 [119,276]. Other intracellular regulators of the Smad pathway are the phosphatases. Protein phosphatase, Mg^{2+}/Mn^{2+} dependent, 1A (PPM1A) dephosphorylates Smad1 and inhibits its BMP-2-induced transcriptional activity [61].
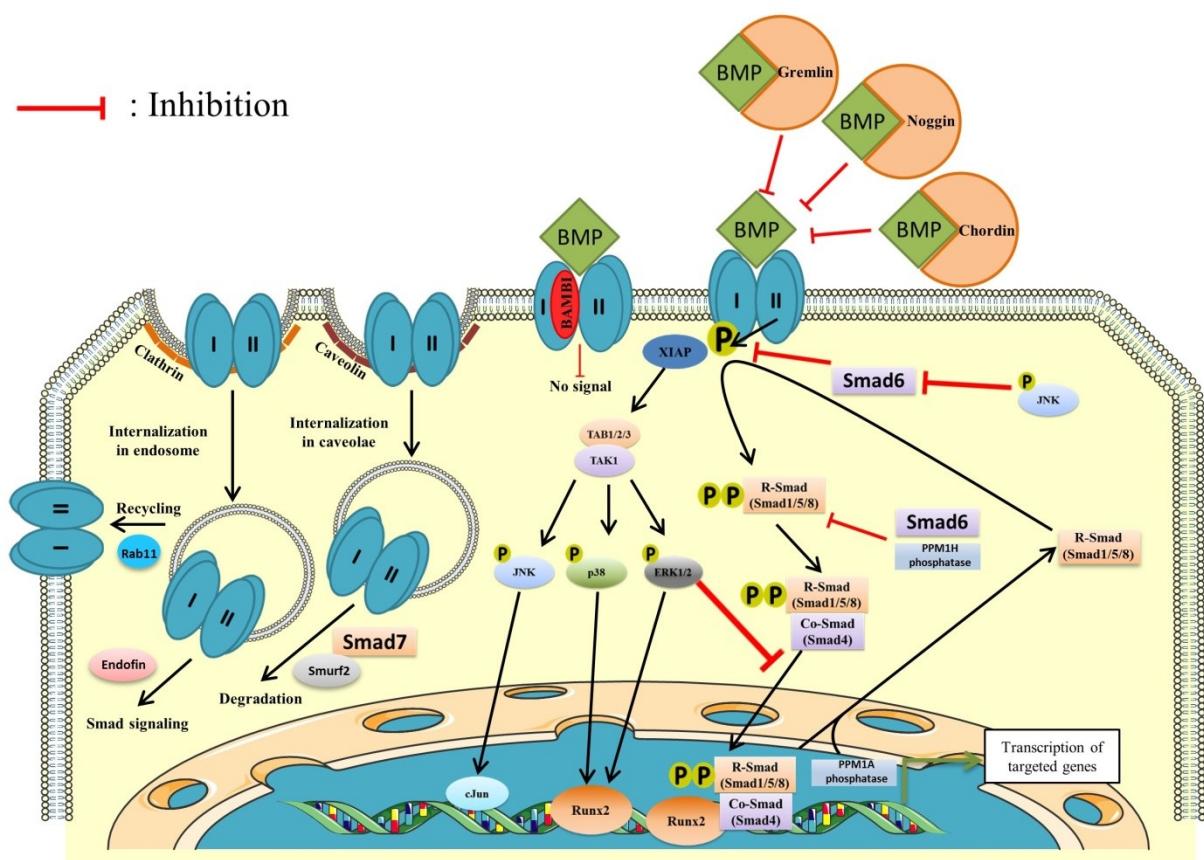
Figure 2.2 Regulation of BMP-induced signaling
[21,39,271,61,147,165,167,189,190,209,269]. [Illustration using Servier Medical Art, http://www.servier.fr]

The interaction of a BMP with its receptors can also activate the MAPK signaling pathway (Figure 2.2). The MAPK pathway is divided into 3 cascades: ERK1/2, p38 and c-jun N-terminal kinase (JNK). The BMPs facilitate the recruitment of a MAPKKK, TAK1, to the

type I receptor and then the activation of the 3 MAPK cascades [271]. The mechanism by which TAK1 is activated by type I receptors is still unknown. Perhaps the X-linked inhibitor of apoptosis (XIAP) mediates the signal transduction between BMP receptor and TAK1 [323]. The phosphorylated ERK1/2, p38 and JNK are then translocated to the nucleus, where they interact with the factors controlling the transcription of specific genes [167]. ERK1/2, p38 and JNK can have either positive or negative effect on osteoblastic differentiation. Xu *et al.* showed that inhibiting p38 decreased the synthesis of ALP by C3H10T1/2 cells infected with AdBMP-9 [318]. Similarly, Lauzon *et al.* found that the inhibition of either JNK or ERK1/2 increased ALP activity in MC3T3-E1 preosteoblasts stimulated with BMP-9 or BMP-2 in the presence of fetal bovine serum [153]. Others have found that ERK1/2 regulates the Smad canonical pathway by phosphorylating Smad1 at the linker region, so preventing the complex formed by Smad1 and Smad4 from being translocated to the nucleus [147].

BMPs and other growth factors can act in synergy to trigger the differentiation of MSCs or bone cells. Lauzon *et al.* showed that IGF-2 increased the BMP-9-induced ALP activity in MC3T3-E1 cells [153]. FGF2 (or bFGF) is also involved in osteogenic differentiation through its action on the concentration of BMP-2. The concentration of BMP-2 in FGF2$^{-/-}$ mice is drastically decreased leading to reduced bone formation [198]. Epidermal growth factor (EGF) also enhances the ALP activity in immortalized mouse embryonic fibroblasts that have been infected with AdBMP-9 [166].

The canonical Wnt/beta-catenin signaling pathways, Notch and Hedgehog (Hh) are also crucial for the differentiation of MSCs into osteoblastic lineage (Figure 2.3) [33,172]. The Wnt/beta-catenin pathway is important for determining the fate of stem cells; it not only favors osteogenic differentiation, it also inhibits adipogenic differentiation [31]. Canonical Wnt agonists act on cells by binding to its receptor Frizzled (Fzd) and its co-receptors low density lipoprotein receptor related protein 5 and 6 (LRP5/6). This leads to the recruitment of the Dishevelled (Dvl) protein, which inhibits the phosphorylation of the beta-catenin by glycogen synthase kinase-3 beta (GSK-3 beta) and its subsequent ubiquitination and degradation by proteasomes. The unphosphorylated beta-catenin can then translocate to the nucleus and interact with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF). This complex enables the transcriptional activity of genes encoding proteins like Runx2 and BMP-2

[10,41,80]. Tang *et al.* showed that C3H10T1/2 cells infected with AdWnt-3a increased ALP activity and enhanced the ALP activity induced by AdBMP-9 [293].
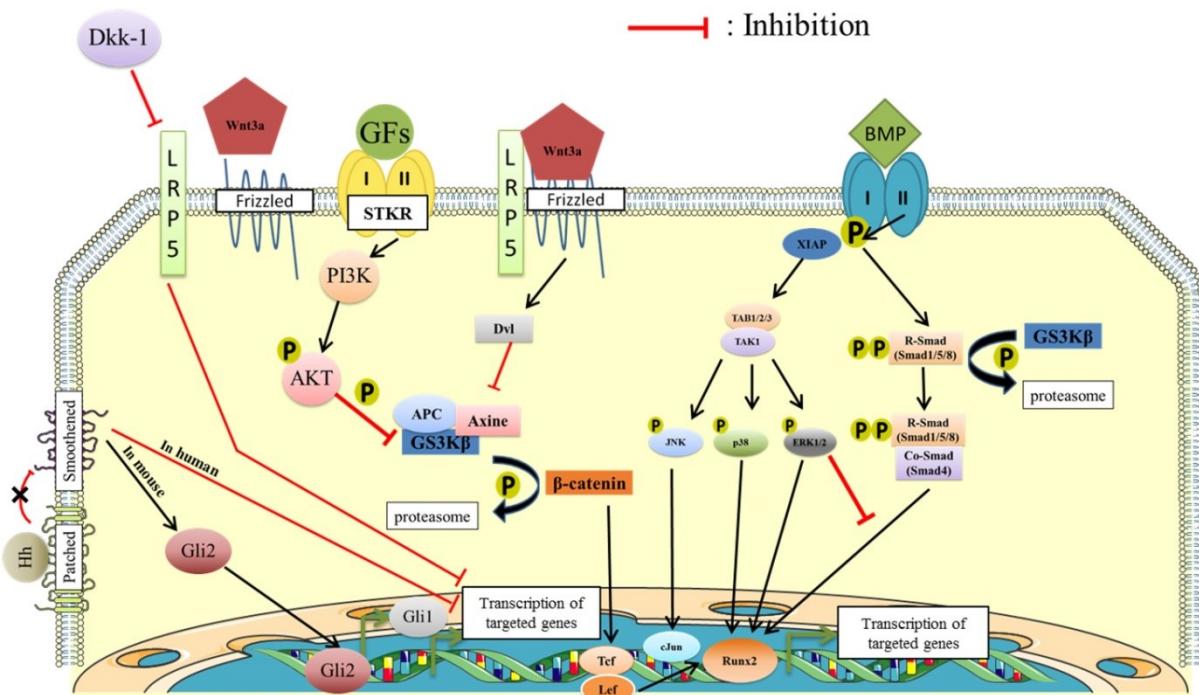
Figure 2.3 Crosstalk between Wnt and growth factors that regulate osteoblast behavior (APC, adenomatous polyposis coli; Dkk-1, Dickkopf-1; GFs, growth factors; STKR, serine/threonine kinase receptor) [10,110,277,147,155,163,167,190,227,250,271]. [Illustration using Servier Medical Art, http://www.servier.fr]

Notch is a transmembrane receptor that interacts with the ligands Delta or Jagged present on the surface of neighboring cells. Notch intracellular domain (NICD) is then cleaved by gamma-secretase and moves to the nucleus, where it binds to transcription factors like CBF1, suppressor of hairless (CSL) and the co-activator Mastermind-like (MAML) to stimulate the transcription of genes encoding Hairy enhancer of split (Hes) and Hey [163]. Ugarte *et al.* showed that activating the Notch pathway in human MSCs by causing them to overproduce Jagged1 or NICD, induced mineralization and increased their ALP activity and BMP-2 expression, while inhibiting their differentiation into adipocytes [299]. However, activating the Notch pathway does not stimulate OC synthesis, which suggests that the Notch pathway induces early osteogenic differentiation but not the formation of mature osteoblasts. The transcription factors like Hes and Hey, which are Notch targeted genes, also influence the

responses of cells to BMPs. Sharff *et al.* observed that silencing Hey1 in C3H10T1/2 cells reduced their BMP-9-mediated ALP activity. Infecting the cells with AdRunx2 caused the ALP activity to recover [263]. BMP-2 also triggers C2C12 cells to undergo osteogenic differentiation by increasing the expression of genes encoding for ALP and OC, together with increased expression of gene encoding for Hey1 and decreased Hes1 transcripts [52]. However, Zamurovic *et al.* found that Hey1 antagonized the transcriptional activity of Runx2 in MC3T3-E1 preosteoblasts stimulated by BMP-2, which led to decreased mineralization [333].

Hedgehog (Hh) is also involved in osteogenic differentiation. It binds to its receptor Patched at the cell surface, which prevents Patched from inhibiting the transmembrane protein Smoothened. This enables Smoothened to activate a signaling cascade leading to stabilization of the transcription factor Gli2. The newly-stabilized Gli2 then activates the transcription of target genes such as that encoding Gli1, which is also a transcription factor that promotes the expression of genes like those encoding ALP and BSP [107,163]. The effect of Hh on differentiation depends on the species. Plaisant *et al.* observed that conditioned medium from sonic Hh-secreting cells inhibits the synthesis of ALP, Runx2, osteonectin and OPG by human multipotent stem cells derived from adipose-tissue [227]. There is also a crosstalk between Hh and the Wnt pathway. Hu *et al.* showed that inhibiting the Wnt pathway in C3H10T1/2 cells by transfecting the cells with retrovirus encoding Dkk-1 decreased the ALP production induced by a constituvely active Smoothened protein [110]. Spinella-Jaegle *et al.* found that stimulating C3H10T1/2 cells with sonic Hh increased the ALP activity induced by BMP-2. However, sonic Hh had no effect on the ALP activity in MC3T3-E1 preosteoblasts induced by BMP-2 [277].

2.5.2 Osteoclastogenesis

Osteoclastogenesis involves the commitment of hematopoietic precursor cells to the monocyte/macrophage lineage, the fusion of several precursors and their transformation into mature osteoclasts [20]. These processes are regulated by two major signaling pathways that are activated by macrophage colony-stimulating factor (M-CSF) and RANKL, a member of the tumor necrosis factor (TNF) ligand superfamily. M-CSF promotes RANK expression and mediates the proliferation of osteoclast precursors and their differentiation and survival.

RANKL is crucial for osteoclast differentiation, survival and bone-resorbing activity (Figure 2.4) [105,298,308]. RANKL also favors the retention of the osteoclast precursors in bone by down-regulating the gene encoding the receptor S1PR1 of the lipid mediator sphingosine-1 phosphate (SP-1), which favors the passage of osteoclast precursors from the bone to blood vessels [121]. M-CSF and RANKL are synthesized by osteoblasts, osteocytes, bone marrow stromal cells and lymphocytes in response to stimulation by factors including hormones (parathyroid hormone [PTH], vitamins D), inflammatory cytokines (Interleukin-1 [IL-1], IL-6, TNF alpha, interferon-gamma, IFN gamma) [201,225,289,312].
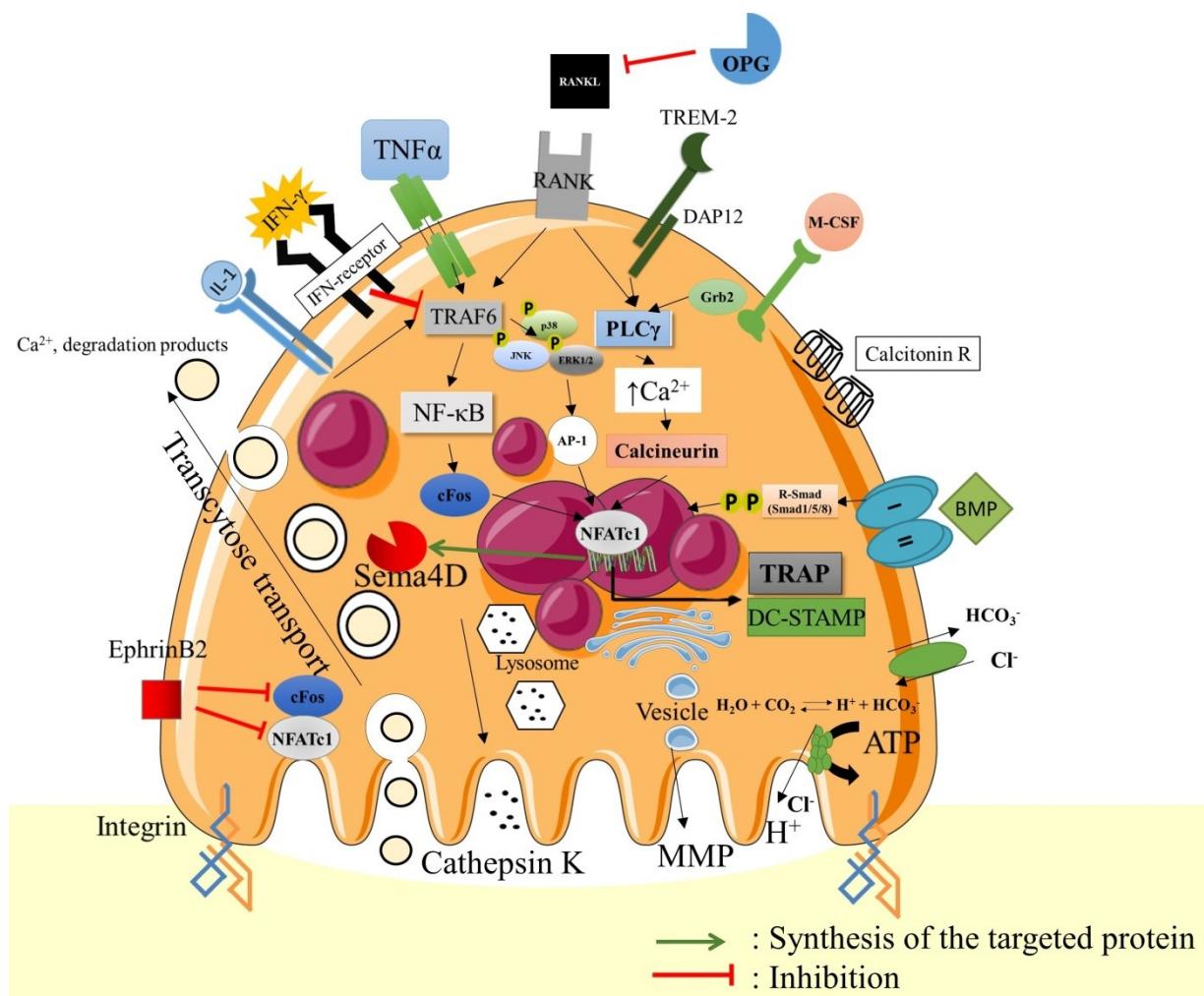
Figure 2.4 Osteoclast structure and regulation by cytokines and growth factors (AP-1, activating protein-1; PLCgamma, phospholipase C gamma; TRAP, tartrate resistant acid phosphatase) [20,144,149,170,203,289,302,327,337]. [Illustration using Servier Medical Art, http://www.servier.fr]

M-CSF binds to the tyrosine kinase receptor c-Fms on osteoclast precursors and causes activated c-Fms to form dimers. These become phosphorylated on their multiple tyrosine residues, enabling them to interact with proteins containing SH2 domains like Grb2 (Y697, Y974), c-Src (Y559), PI3K (Y721) and to transduce intracellular signaling of M-CSF. PI3K then stimulates Akt, while Grb2 activates the Ras/Raf/MEK/ERK pathways by interacting with Sos (for review see [242,319]). These pathways are mainly involved in the proliferation and survival of osteoclast precursors. However, Amano *et al.* recently demonstrated that the M-CSF-triggered differentiation of murine 4B12 precursor cells (Mac-1 (+) c-Fms (+) RANK (+) cells from calvaria of 14-day-old mouse embryos) into osteoclasts also depended on the activation of ERK5 [6].

RANKL acts on osteoclast precursors by binding to its RANK receptor, which, in turn, allows the binding of TNF receptor associated factor-6 (TRAF6) to the intracellular domain of RANK. It induces the activation of several signaling pathways including NF-kappaB, MAP kinases (JNK, ERK1/2, and p38), and also leads to the mobilization of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [20,327]. RANKL may also activate the calcium signals that lead to the activation of another major transcription factor, nuclear factor of activated T cells cytoplasmic 1 (NFATc1), through an immunoreceptor tyrosine-based activation motif-mediated co-stimulatory signaling [273]. RANKL-RANK binding induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to give inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (for review see [208]). The subsequent release of calcium ions from the endoplasmic reticulum induced by IP3 activates calcineurin, which then stimulates NFATc1 by dephosphorylating it [290]. NFATc1 activates genes encoding proteins essential for osteoclastogenesis, such as dendritic cell-specific transmembrane protein (DC-STAMP), which is required for cell fusion [149]. The activation of NFATc1 by RANKL is amplified by stimulating osteoclast precursors with immunoglobulin-like receptor ligands that bind triggering receptor expressed in myeloid cells 2 (TREM-2) or osteoclast-associated receptors (OSCAR). These receptors become activated by associating with adaptor proteins containing the immunoreceptor tyrosine-based activation motif (ITAM). The ITAM-mediated signal from TREM-2 involves the 12 kDa DNAX-activating protein (DAP12), while OSCAR involves the Fc receptor common gamma [144,288]. The adaptors recruit the protein tyrosine kinase Syk, and the resulting signal leads to the release of calcium and activation of

transcription factors like NFAT and NF-kappaB. Barrow *et al.* recently reported that OSCAR can bind strongly to type I collagen [11]. This interaction promotes osteoclastogenesis [11]. The effects of pro-osteoclastogenic factors may also depend on signaling modulators. The recombinant recognition sequence binding protein at the Jkappa site (RBP-J) is a novel transcription factor which inhibits osteoclastogenesis by imposing a requirement of ITAM-mediated co-stimulation for TNF alpha and RANKL signaling [161]. Activating RBP-J in osteoclast precursors suppresses the osteoclastogenesis induced by inflammatory stimuli, such as TNF alpha. Conversely, inactivation of RBP-J considerably increases RANKL-independent osteoclastogenesis [335]. Finally, the fine-tuning of bone resorption also involves OPG, a secreted decoy RANKL receptor that competes with RANK and inhibits osteoclast differentiation and functions. RANKL signaling can be completely blocked by sequestering RANKL using OPG [20,327]. It is also inhibited by cytokines such as IFN gamma [289].

Osteoclastogenesis is also regulated by BMPs like BMP-2 [22,122,126]. Thus, the differentiation of bone marrow cells into osteoclasts requires less RANKL (half optimal dose) when BMP-2 is present in the culture medium [126]. The extracellular inhibitor of BMP-2, Noggin, severely impairs the osteoclastogenesis induced by RANKL and the BMP type II receptors BMPRII is required for osteoclastogenesis *in vitro* [22,126]. Using human mononuclear leukocyte suspensions isolated from umbilical cord blood, Fong *et al.* showed that BMP-9 could protect osteoclasts against apoptosis via a decrease in caspase-9 activation [72]. BMP-9 also enhanced the bone resorption by mature osteoclasts [72].

2.5.3 Bone healing

Bone healing can be divided into two processes, one not excluding the other: intramembranous and endochondral healing. Intramembranous process occurs in fractures that heal by first intention. Fracture sites should have no defect and should be mechanically stable (described in more details in the next section). The healing of larger bone fractures involves both endochondral bone formation and intramembranous healing. Endochondral healing (second intention) occurs in four phases: inflammation and hematoma formation, fibrocartilagenous callus formation, bony callus formation, and finally bone remodeling (for review see [64]). The periosteum is important for bone healing especially during endochondral healing since it provides chondrogenic lineage precursor cells [219]. Each phase requires

different cytokines, growth factors and vitamins. Growth factors like BMP, TGF-beta, IGF, FGF, PDGF and VEGF all are involved in bone repair (Figure 2.5) [3]. The PTH is also important in bone healing process since it can enhance bone formation by increasing cartilage volume as well as bone mineral density (for review see [64]).

Inflammation	Chondrogenesis	Osteogenesis	Late Osteogenesis
IL-1, IL-6	**IL-6**	**IL-6**	**IL-1, IL-6**
BMP-2, TGF-β	**BMP-2, BMP-5, BMP-6,**	**BMP-2, BMP-3, BMP-4,**	**BMP-2**
GDF-8	**BMP-14**	**BMP-7, BMP-8**	**RANKL, M-CSF**
Prostaglandin E2	**TGF-β2, TGF-β3**	**RANKL, M-CSF**	**Vitamin D**
PDGF	**VEGF, Angiopoietin**	**VEGF**	
FGF-1, FGF-2, FGF-5	**IGF**	**IGF**	
Vitamin D	**FGF-1, FGF-2, FGF-5,**	**TNF-α**	
	FGF-16, FGF-18	**FGF-1, FGF-2, FGF-9,**	
	Vitamin D	**FGF-16, FGF-17, FGF-18**	
		Vitamin D	

Hematoma formation → Fibrocartilagenous callus formation → Bony callus formation → Bone remodelling

Figure 2.5 Endochondral healing process following bone fracture [3,60,64,89,326,329]. [Illustration using Servier Medical Art, http://www.servier.fr]

Several types of cells are also involved in the endochondral bone healing. Macrophages are recruited during hematoma formation/inflammation to remove damaged cells and tissue. They secrete cytokines that foster the recruitment (infiltration) and stimulation of leukocytes to the injury site [78,256]. Prostaglandin E2, which is present at the fracture site, can also recruit T lymphocytes during this step [54]. The second phase of bone healing is the formation of the fibrocartilagenous callus. The chondrogenic lineage precursor cells in the periosteum move to the injury site, where they differentiate into mature chondrocytes under the influence of BMP-2, which is present throughout bone healing [329]. Other cytokines and

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growth factors also promote the differentiation of the precursor cells into mature chondrocytes. TGF-beta3 also facilitates the differentiation of stem cells into chondrogenic lineage (TGF-beta3-BMP2 synergy) [268]. FGF-2 can enhance and control the proliferation of the chondrocytes and osteoblast progenitors during each of the bone healing step [60]. The transition between the fibrocartilagenous and bony callus phases is promoted by angiogenesis and the differentiation of precursor cells into mature osteoblasts. VEGF and angiopoietin are involved in the vascularization of the callus and the final bone formation/mineralization [64].

Finally, the last stage of bone healing is bone remodeling, which can be subdivided in five distinct phases: quiescence, preosteoclast recruitment and osteoclast differentiation, bone resorption, preosteoblast recruitment and osteoblast differentiation which ultimately lead to the formation of structured bone. The first step is osteoclastogenesis and two hypotheses have been advanced to explain osteoclast activation. The first is that osteoclastogenesis is activated by the apoptotic bodies of the osteocytes while the second is that the death of osteocytes leads to a reduction in the amount of TGF-beta [100,101,145]. The activated osteoclasts then start to resorb bone [302,337]. Resorption takes up to 30 days, while bone formation and the recruitment of osteoblasts and osteoblast precursors can take 150 days. The cell signaling that triggers the passage from bone resorption to formation is not yet fully understood. Mature osteoblasts then synthesize the bone matrix and allow its mineralization.

The various cells involved in bone remodeling communicate with each other in several ways throughout the process. Osteoclasts synthesize Sema4D that inhibits the formation of the bone matrix by osteoblasts [203]. Sema4D binds to the Plexin-B1 receptors on the osteoblasts and these then activate RhoA to prevent the matrix mineralization induced by IGF-1 [203].

2.6 Cell adhesions

2.6.1 Types of cell adhesions

Interactions between cells and ECM or biomaterials are essential for tissue integrity and repair. They influence cell survival, proliferation, differentiation and migration. The integrins are the main heterodimeric alpha beta transmembrane glycoproteins cell receptors involved in the adhesion of cells to ECM proteins and biomaterials [118]. There are presently 18 alpha and 8 beta subunits that assemble to form 24 distinct integrins. They interact with

adhesive proteins from the ECM via specific amino acid sequences. The most common site on ECM proteins to which integrins bind is the tripeptide sequence Arg-Gly-Asp (RGD). It is found on type I collagen, OPN, BSP, thrombospondin and fibronectin [245]. Alphav beta3 integrins bind to several proteins, including fibronectin, vitronectin and BSP, while alpha5 beta1 integrins interact specifically with fibronectin. Collagen type I also interacts with alpha1 beta1 and alpha2 beta1 integrins [118,127].

Osteoblasts

Osteoblasts can bear alpha1, alpha2, alpha3, alpha4, alpha5, alpha6, alphav, beta1, beta3 and beta5 integrin subunits depending on their stage of differentiation. Alpha2, alpha3, alpha5, alphav, beta1 and beta3 subunits are present on osteoblasts, alpha2 integrin subunit is found only on cells close to the bone surface while alphav beta3 integrins are found mainly on osteocytes [207].

Osteoblasts can adhere to 2D systems *in vitro* via integrin adhesion architectures that are organized as focal complexes, focal adhesions and fibrillar adhesions, but the morphology, size and subcellular distributions of these cell adhesion sites differ [330]. Focal complexes that are 0.1 to 2 microns in diameter are the main precursors of focal adhesions. They recruit vinculin and phosphoproteins and exert stress between 1 and 3 nN/micron^2. Focal adhesions are elongated streak-like structures (3 to 10 microns) that are often located at the cell periphery [260]. They anchor bundles of actin stress fibers (F-actin) through a plaque made up of several proteins including integrins and structural proteins. Integrin-mediated adhesions called adhesomes are composed of more than 180 components [331]. Adhesomes contain actin-associated proteins (tensin, vinculin, alpha actinin), adaptor proteins (vimentin, Shc-transforming protein 1) and signaling proteins (tyrosine or serine/threonine kinases and members of the Rho GTPases) [117]. Three proteomic studies on the composition of adhesomes in cells attached to fibronectin and control cells on matrices like poly-L-lysine have identified more than 700 components [113,150,257]. These studies also found new focal adhesive proteins such as actin linkers (adducing) and ubiquitin ligase proteins. However, only 63 proteins associated with integrin-mediated adhesion are common to all three studies perhaps because of the cell types and substrata used [84,113,150,257]. Robertson *et al.* recently used proteomic and phosphoproteomic methods to analyze the components of

integrin-mediated adhesions that attached A375-SM human melanoma cells to polystyrene (PS) surfaces coated with fibronectin or control PS surfaces coated with transferrin [240]. They found more than 1170 proteins in adhesomes, including 499 phosphoproteins. Hoffman *et al.* also recently used fluorescence cross-correlation spectroscopy and fluorescence recovery after photobleaching and found that some components of the adhesome existed as cytoplasmic pre-assembled complexes that were available for rapid assembly and adhesion site formation [106]. Clearly, further investigations are required to give a better picture of the composition and how the adhesome is assembled.

The formation of focal adhesion sites depends on the activation state of the integrins, and this implies a change in their conformation (Figure 2.6). The binding of structural proteins such as talin to the cytoplasmic tail of the beta integrin subunits allows the integrin head region to be repositioned to point away from the cell surface. The cytoplasmic domain of each beta integrin subunit is therefore a major factor in establishing the connections between the cell cytoskeleton and the ECM. Integrin-linked kinases (ILK) that interact with beta1 integrin subunits are also central to integrin signaling [65]. The interaction of talin with vinculin then promotes the clustering of activated integrins [114]. The cytoplasmic domains of beta integrin subunits also contain one or two conserved Asn-Pro-X-Tyr, or Asn-Pro-X-Phe motifs that interact with phosphotyrosine proteins such as focal adhesion kinase (FAK) [264]. FAK is a non-receptor protein tyrosine kinase that possesses three domains, an N-terminal ezrin radixin moesin (FERM) domain, a central kinase catalytic domain and a C-terminal FAT domain. FERM is involved in protein-protein interactions [74,254,258] (for review see [186]). It interacts especially with the phenylalanine 596 in the central catalytic domain of FAK, thus inhibiting the kinase [162]. The FAK kinase contains three tyrosines (Y397, Y576 and Y577); their phosphorylation is essential for the activation of FAK [296]. Integrin clustering first causes the rapid autophosphorylation of FAK at Y397. FAK phosphorylated on Y397 then interacts with proteins like the Src-family kinases that phosphorylate other tyrosine residues in FAK such as the two tyrosine residues in the catalytic loop of the kinase domain (Y576 and Y577) so increasing the FAK kinase activity. These kinases also phosphorylate two proteins that interact with FAK, Crk-associated substrate (CAS) and paxillin [255]. Paxillin contains many tyrosine and serine/threonine phosphorylation sites and is believed to modulate both cell adhesion and growth factor signaling pathways [262]. Paxillin activates some members of the

RhoGTPases family such as Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42) by recruiting Pak-interacting exchange factor and guanine exchange factor (GEF). These members of the RhoGTPases family are involved in cell adhesion and organization of the cytoskeleton [117]. Their activation depends on GEF catalyzing the exchange of a GDP for a GTP on the RhoGTPases proteins. Inactivation of the RhoGTPases is mediated by a GTPase activating protein (GAP) that favors the hydrolysis of GTP to GDP. FAK-Src complex acts via p190RhoGAP to keep a RhoGTPase called RhoA inactive during the early steps of cell adhesion and spreading. In contrast, Cdc42 and Rac1 are active: Cdc42 regulates the formation of filopodia while Rac1 regulates membrane ruffling lamellipodia. RhoA becomes activated by GEFs like p115RhoGEF and p190RhoGEF at a later step in cell spreading, which favors the formation of actin stress fibers and the maturation of focal adhesions. Stimulation of RhoA suppresses the activity of Rac1 [117].
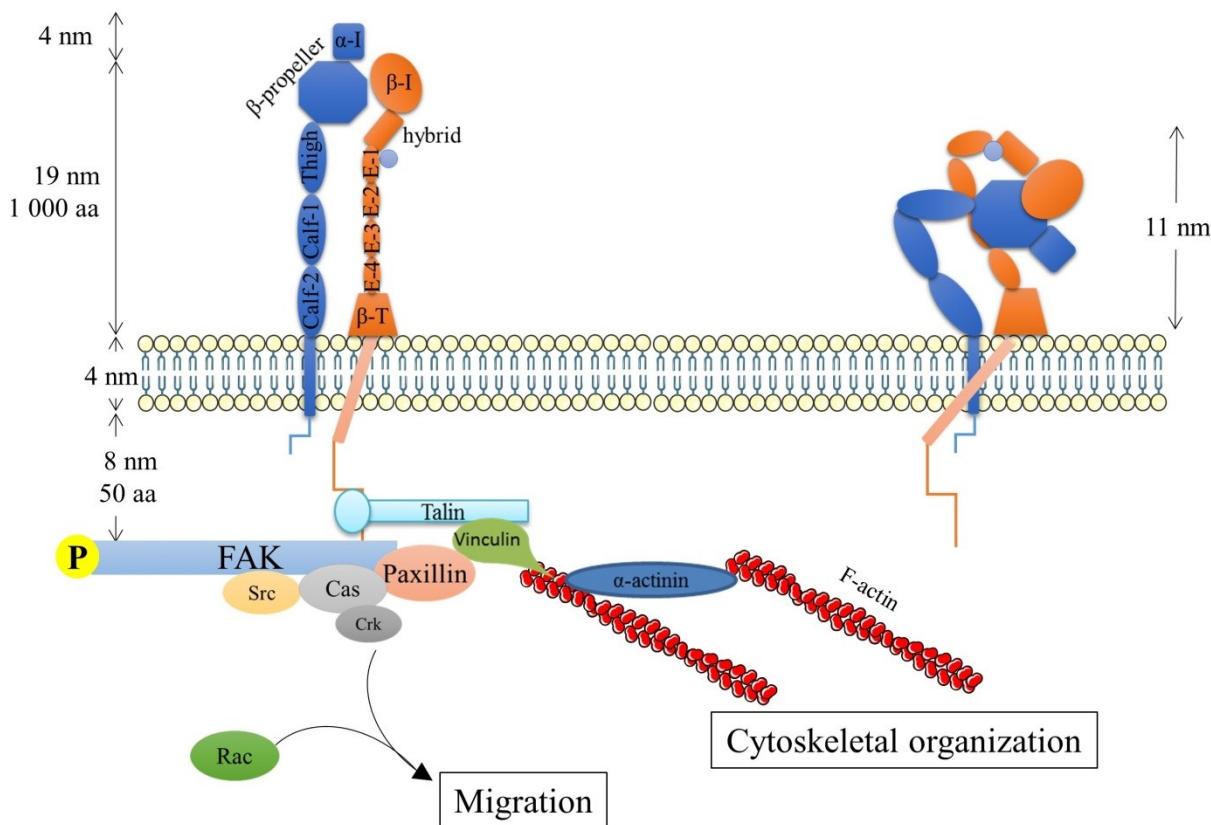
Figure 2.6 Activation of integrin and focal adhesion organization (E1, Epidermal Growth Factor domain 1; Beta T, beta tail) [49,82]. [Illustration using Servier Medical Art, http://www.servier.fr]

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Fibrillar adhesions, consisting of tensin-alpha5 beta1 integrin complexes that bind to fibronectin, are more centrally located in the cell than are focal adhesions. The formation of fibrillar adhesions is closely linked to the capacity of cells to polymerize fibronectin to form ECM fibrils [136,332]. The translocation of fibrillar adhesions is highly directional, proceeding centripetally from the cell periphery towards the center and is always aligned along the long axis of the focal adhesion. Multi-ligand alphav beta3 integrins remain within focal adhesions, while alpha5 beta1 integrins are translocated at 6.5 microns/h parallel to the actin microfilaments in fibrillar adhesions [136,332]. Lin *et al.* recently reported that osteoblasts at 12 hours organize endogenous fibronectin into fibrils on self-assembled monolayers bearing amine groups, while those bearing hydroxyl and methyl groups limit such fibronectin fibril formation [164].

However, it is known that the components of 2D and 3D adhesions differ considerably: 3D adhesions used mainly alpha5 beta1 integrins instead of alphav beta3 and had a low FAK phosphorylation [322].

Osteoclasts

Osteoclasts contain alpha2, alpha5, alphav, beta1 and beta3 integrin subunits. However, the adhesion receptor alphav beta3 is the major integrin expressed by osteoclasts and a marker of the osteoclast phenotype. The engagement of alphav beta3 integrin leads to the formation of a multi-molecular complex that includes c-Src and PI3K [88]. In addition to its role in cell-matrix attachment, alphav beta3 is also involved in other aspects of osteoclast biology, and osteoclasts that lack alphav beta3 are dysfunctional [68,241]. The bone matrix mediates anti-apoptotic signals via integrins, and alphav beta3 occupancy promotes osteoclast survival [77]. In contrast, unoccupied alphav beta3 sites may induce osteoclast apoptosis via caspase-8 activation, and in the absence of alphav beta3 (beta3$^{-/-}$ knockout mice), delayed cell death occurs as a result of caspase-9 activation [283,338].

Upon adhesion, osteoclasts reorganize their cytoskeleton and form podosomes which are small adhesion structures (1 micron diameter) with a dense actin core surrounded by a rosette-like structure containing proteins such as alphav beta3 integrins, structural focal adhesive proteins (talin and vinculin), actin-associated proteins (gelsolin and alpha-actinin), tyrosine kinases (c-Src and Pyk2) and small GTPases. RhoGTPases have been involved in the

organization of F-actin cytoskeleton in different cell types, and in mature osteoclasts Rac, Cdc42 and Rho regulate the podosome formation into the sealing zone [248,253,291]. The podosome core also contains large amounts of proteins involved in actin polymerization. Thus, the Arp2/3 complex that is activated by Wiscott-Aldrich syndrome protein (WASP) takes part in the formation of the actin podosome core, while the formin protein initiates the unbranched F-actin strands and the formation of stress fibers [109]. Cortactin, which interacts with the Arp2/3 complex, is also involved in the formation and stabilization of the branched actin network [301]. By contrast, formation of the ruffled membrane involves vesicular trafficking regulated by the Rab family of GTPases, particularly lysosomal Rab7 [123].

Osteoclasts are capable of adhesion on different substrates by distinct F-actin structures. When osteoclasts adhere on plastic or glass, podosomes form clusters organized in a podosome belt around the cell, in which podosomes can be individualized (Figure 2.7). Upon adhesion to bone or mineralized ECM, podosomes undergo major reorganization as part of the osteoclastic terminal differentiation into bone resorbing cells. In non-active (non-resorbing) unpolarized osteoclasts, podosomes are dispersed on the adhesion surface or organized in a loose belt of podosomes [123]. When osteoclasts are activated to resorb bone, they strongly attach to the bone matrix by densely packed F-actin rich podosomes to form the sealing zone that delineates the cellular area in contact with the bone surface where the ruffled membrane enlarges and beneath which the resorption lacuna occurs [55,211]. Some studies suggest that a podosome belt prefigures the sealing zone but this is rather controversial [55,129,173,297]. Microtubules, made up of alpha- and beta-tubulin heterodimers in filamentous networks, also regulate the podosome organization at the end of osteoclast maturation [55]. Podosomes are also involved in the migration of osteoclasts, perhaps through a saltatory mode [111]. Touaitahuata *et al.* suggested recently that adhesion is essential for osteoclast differentiation while podosome formation is not [297].
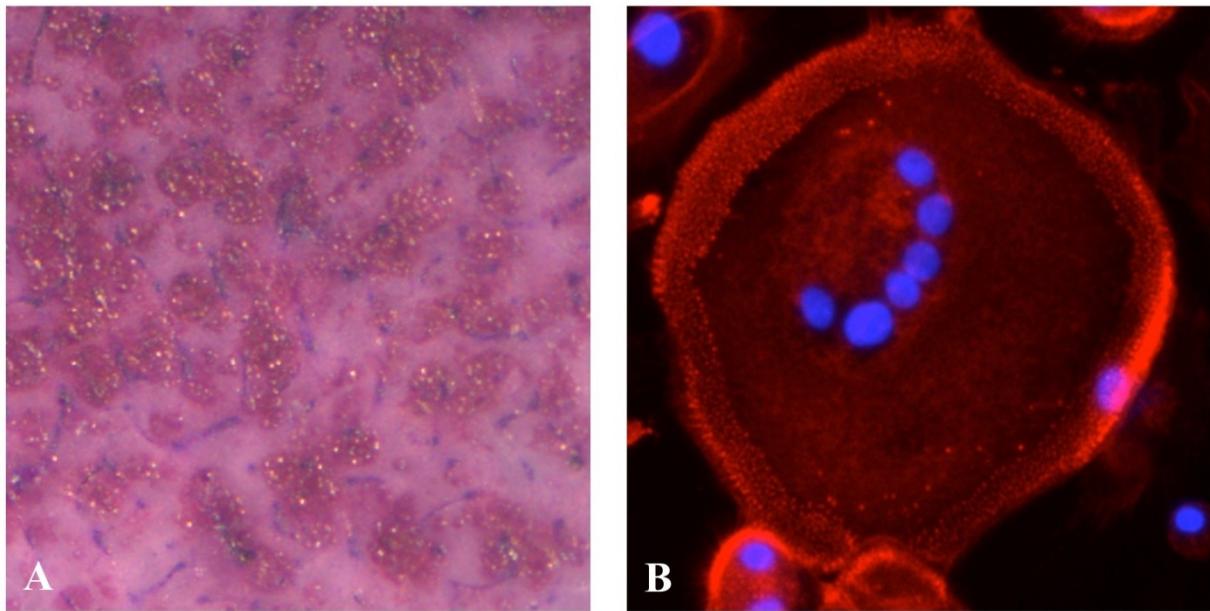
Figure 2.7 Bone resorption and actin ring formation. Mature osteoclasts were obtained from cord blood monocytes in long-term cultures in presence of MCSF and RANKL. The cells were allowed to settle either on devitalized bovine bone slices or plastic. (A) Bone resorption appears as dark areas after toluidine blue staining, with a bright aspect under epi-illumination. (B) Cell cultured on plastic and F-actin, found in actin ring, was stained with fluorescent-labeled phalloidin (red) and nuclei with DAPI (blue).

2.6.2 Influence of ECM-integrin interactions on cell behavior

Integrins that are involved in the interactions between cells and the ECM may also influence the responses of cells to growth factors such as BMPs, VEGFs, PDGFs and IGFs [66,87,151,236]. Integrins and growth factors influence each other in many ways. Integrins can be co-localized with growth factor receptors. Lai and Chen demonstrated that specific integrin subunits (alphav, beta1, beta3, alphav beta3, alphav beta5, alphav beta8) were located with type I and type II (BMPRI and BMPRII) BMP receptors [151]. They also showed that blocking the alphav integrin subunits with antibodies decreased the ALP activity induced by BMP-2. Goel *et al.* found that IGFIR was located together with beta1A integrins at focal adhesion points in GD25 cells [87]. Interactions that involve the insulin receptor substrate-1 (IRS-1) maintain IGFIR in an activated state that favors cell proliferation and tumor growth. In contrast, beta1C integrin subunits that form a complex with Gab1/Shp2 promote the recruitment of Shp2 to IGFIR, which then leads to dephosphorylation of the receptor. This inactivation of IGFIR slows down cell proliferation and thus inhibits tumor growth [87].

Integrin also act on growth factor signal transduction by recruiting adaptors to the plasma membrane or specific signaling proteins involved in the adhesome. Integrins can induce the phosphorylation of growth factor receptors in the absence of their ligand [304]. Veevers-Lowe *et al.* found that the PDGF receptor beta in human bone marrow MSCs attached to fibronectin via their alpha5 beta1 integrins was already phosphorylated, without any growth factor stimulation [304]. This activation is essential for MSC migration [304]. The N-terminal FERM domain of FAK also interacts with growth factor receptors such as those of PDGF [35,104,128,304]. In addition, Tamura *et al.* demonstrated that FAK was essential for the differentiation of MC3T3-E1 preosteoblasts induced by recombinant human BMP-2 [292]. No ALP activity was detected in FAK-deficient cells treated with BMP-2.

These versatile integrins also modulate the internalization and degradation rate of growth factor receptors. Reynolds *et al.* showed that the alphav beta3 inhibitor stimulated tumor growth and tumor angiogenesis by recycling the VEGFR2 internalized in endosomes back to the plasma membrane and by decreasing VEGFR2 degradation [236]. Finally, integrins stimulate the synthesis of growth factors. Mai *et al.* reported recently that silencing the gene encoding for the beta1 integrin subunit inhibited the synthesis of BMP-2 and the differentiation of MC3T3-E1 preosteoblasts into osteoblasts by preventing activation of the gene encoding ALP [176].

However, growth factors also regulate the synthesis of specific integrins. Mena *et al.* observed that stimulating human umbilical vein endothelial cells (HUVECs) with VEGF-A increased the production of alpha5 and beta1 integrin subunits [184]. Similarly, our research group has shown that a peptide derived from the knuckle epitope of BMP-9 increased the amount of alphav integrin subunits in the plasma membrane of MC3T3-E1 preosteoblasts attached to PS coated with peptides derived from BSP [179]. Lai and Chen also showed that stimulating human osteoblasts (HOB19) with BMP-2 led to the synthesis of alphav, beta1 and beta3 integrin subunits in human osteoblasts [151]. As BMP-2 increased the amount of alphav beta3 integrins in human osteoblasts, it favored their adhesion to OPN and vitronectin [151].

The crosstalk between integrin and growth factor signaling pathways coordinates MSCs and bone cells behaviors. Researchers that develop biomimetic materials must therefore be aware of such complex interactions. They have to get a better understanding of the

influence of biomimetic materials on the ability of cells to respond to growth factors, cytokines and hormones.

2.7 Biomimetic materials

2.7.1 Materials functionalized with proteins

The biomaterials used in bone repair can be inorganic materials, natural polymers, synthetic polymers, or even composites. But not all bone biomaterials currently in use are osteoconductive and osteoinductive (for review see [160,180]). Biomaterials can be functionalized with ECM proteins such as fibronectin, collagen, OPN and BSP to increase their interactions with cells [231,259]. The proteins can be immobilized on the surfaces by non-specific adsorption (physisorption) or by covalent binding (for review see [180]). Schwab *et al.* tested fibronectin, vitronectin and OPN adsorbed onto PS. Fibronectin and OPN promoted greater hMSC proliferation in comparison with the control after 5 and 10 days in standard growth medium [259]. In addition, MSCs attached to fibronectin in osteogenic medium contained more BSP and Runx2 after 5 days, while MSCs attached to OPN contained these markers only after 10 days in culture. Cells on vitronectin contained slightly more osteogenic markers, Runx2 and BSP, after 10 days in osteogenic medium than did cells in standard growth medium. Fibronectin and OPN induced MSCs in osteogenic medium to deposit more calcium than did cells on vitronectin [259].

Several studies have shown that the density and conformation of the proteins adsorbed or grafted onto the surface can strongly affect cell behavior [26,69]. Faia-Torres *et al.* used a gradient of fibronectin (48-213 ng/cm²) on PCL surfaces and showed that human bone marrow MSCs incubated with intermediate densities of fibronectin for 7 days proliferated most rapidly. On the other hand, cells incubated for 7 and 14 days in lower and higher densities of fibronectin had more ALP activity and collagen type I synthesis than did hMSCs on intermediate fibronectin densities [69]. Lin *et al.* also used self-assembled monolayers with terminal $-OH$, $-CH_3$, and $-NH_2$ groups to evaluate the conformation of adsorbed fibronectin [164]. They showed that fibronectin adsorbed onto self-assembled monolayers with terminal -OH groups had more accessible cell-binding domains than did the fibronectin on surfaces with terminal -CH_3 and -NH_2 groups. The more accessible cell binding domains of fibronectin adsorbed onto surfaces with terminal -OH groups led to greater amount of vinculin and tensin

and favored initial cell adhesion at 2h as shown by an increased area of focal and fibrillar adhesions in primary osteoblasts from rat calvaria [164].

The use of proteins extracted from the ECM presents other challenges. There can be batch-to-batch variations [266]. ECM proteins which are extracted and purified from non-human species can increase the risk of undesirable immune responses and infections (238). Peptides derived from proteins that contain the sequence recognized by specific cell membrane receptors have therefore been developed (Tableau 2.1). A major advantage of these peptides is that they are readily synthesized and purified, which reduces their production costs [46].

Tableau 2.1 Effect of ECM-derived peptides on the behavior of bone cells

Protein	Peptide sequence	Negative control	Culture system	Effect	References
Laminin	CRARKQA ASIKVAV SADR (IKVAV)		*In vitro* (Adipose derived stem cells (AdSC) from human tissue)	-↑ number of AdSC onto PCL-IKVAV after 24h and 48h compared with PCL alone	[249]
	DLTIDDSY WYRI (alpha2 chain)	Scramble peptide	*In vitro* (Human osteosarcom a cells)	-↑ number of cells on Ti-coated with the peptide after 1 day compared with Ti alone -↑ ALP activity of cells onto Ti-coated with the peptide after 1 day compared with Ti alone. No difference after 3 days (Ti, Ti-coated with positive or negative peptide)	[187]
	DLTIDDSY WYRI (alpha2 chain)	Scramble peptide	*In vitro* (MG63 osteoblast like cells)	-↑ number and area of cells on PS coated plates with laminin/PS coated with the peptide compared with PS	[131]

				coated with BSA/PS coated with a scramble peptide after 1h in serum-free medium -↑ osteogenic markers for differentiation (BSP, OC) of cells on Ti disk coated with the peptide after 7 days compared with Ti alone -↑ ALP activity of cells on Ti disks coated with the peptide after 3 days compared with Ti alone -↑ number of cells on Ti disks coated with the peptide after 1h compared with Ti alone	
		Scramble peptide	*In vivo* (New Zealand white rabbits)	-↑ bone area for Ti implant coated with the peptide after 1 week compared with Ti alone. No difference after 2 and 4 weeks (Ti, Ti coated with positive or negative peptide) -↑ collagen deposition for Ti implant coated with the peptide after 1 week compared with Ti alone. No difference after 4 weeks (Ti, Ti coated with positive or negative peptide)	

				-↑ ALP activity for Ti implant coated with the peptide after 4 weeks compared with Ti alone or Ti with negative peptide	
BSP	CGGNGEP RGDTYRA Y	CGGNG EPRGET YRAY	*In vitro* (MC3T3-E1)	-Cytoskeletal organization of cells on PCL films functionalized with the peptide after 1h compared with PCL alone or with negative peptide in serum-free medium -↑ area of cells on PCL films functionalized with the peptide after 1h compared with PCL alone or with negative peptide in serum-free medium	[57]
	NGVFKYR PRYYLYK HAYFYPH LKRFPVQ		*In vitro* (Human muscle-derived stem cells)	-↑ ALP activity and calcium deposits in a dose-dependent manner of peptide derived from BSP after 14 days in osteogenic medium -↑ mRNAs of osteogenic genes (ALP, type I collagen, OC, Runx2) was a dose-dependent manner of peptide derived from BSP after 14 days in osteogenic medium	[42]
Fibronectin	RGD-PHSRN K3G4RGD S and K3G4PHSR		*In vitro* (MC3T3-E1)	-↑ number of cells on PHSRN-RGD Ti surface after 4h compared with RGD or PHSRN Ti	[36]

				surfaces alone	
	N			-No difference in term of cellular differentiation (ALP activity) after 14 days	
Collagen type I	GGGGDGE ASP		*In vitro* (Primary rat MSCs)	-↑ ALP activity of cells trapped in hydrogel modified with DGEA peptide after 7 days compared with unmodified hydrogel -↑ number of cells in hydrogel modified with DGEA after 30 days compared with hydrogel modified with RGD or a mixture of RGD and DGEA -↑ collagen type I in cells trapped in hydrogel modified with DGEA after 30 days compared with hydrogel modified with a mixture of RGD and DGEA -↑ OC production and mineralization in cells trapped in hydrogel modified with DGEA after 30 days compared with unmodified hydrogel or hydrogel modified with RGD or a mixture of RGD and DGEA	[183]
	GGYGGGP C(GPP)5GF OGER(GPP		*In vitro* (hMSCs)	-↑ migration of cells on GFOGER-coated random PCL nanofiber after 48h	[146]

	)5GPC			compared with uncoated or collagen-coated meshes. No difference between GFOGER- and collagen-coated aligned meshes -↑ ALP activity of cells on GFOGER-coated random meshes after 21 days compared with uncoated or collagen-coated meshes. ↑ ALP activity of cells on GFOGER-coated aligned meshes compared with collagen-coated meshes -↑ calcium deposition by cells on GFOGER-coated random meshes after 21 days compared with uncoated or collagen-coated meshes. No difference between GFOGER- and collagen-coated aligned meshes	
	RGD		*In vitro* (MC3T3-E1)	-↑ ALP activity and calcification on Ti-RGD after 28 days compared with Ti alone in differentiation-inducing medium	[218]
			In vitro (hMSCs)	-↑ actin cytoskeletal organization and paxillin at focal adhesions in cells	[272]

| | | | | onto PLLA/BCP scaffold modified with RGD after 24h compared with unmodified scaffold

-↑ number of cells and their spreading on PLLA/BCP scaffold modified with RGD after 24h compared with unmodified scaffold

-↑ ALP activity after 7 days and mineralization after 14 days in cells onto PLLA/BCP scaffold modified with RGD compared with unmodified scaffold | |
|---|---|---|---|---|---|
| | KRSRGYC | KSRRGYC | *In vitro* (MC3T3-E1) | -↑ focal adhesions, actin fibers and number of cells on KRSR-TiO_2 anodized nanotubes compared with KSRR-TiO_2 anodized nanotubes or nanotubes alone after 4h

-↑ ALP activity and OC, BSP, OPN, Runx2, Osx and ALP mRNAs for cells on KRSR-TiO_2 after 7 and 14 days compared with KSRR-TiO_2 anodized nanotubes or nanotubes alone | [285] |
| | KRSRC | RKSRC | *In vitro* (Primary human osteoblast, | -↑ number of cells onto calcium aluminate functionalized with | [220] |

			NHOsts)	KRSRC compared with calcium aluminate alone or with the negative peptide, from 1 day to 7 days -Spread and elongated cells on calcium aluminate functionalized with KRSRC after 4 days compared with calcium aluminate alone where cells were rounded	
	GGGGGG GKRSR		*In vitro* (hMSCs)	-↑ number of cells on HAP coated with KRSR after 1h compared to HAP alone -Combination with RGD peptide had no effect on the number of cells -Cells slightly spread on HAP coated with KRSR or in combination with RGD after 1h compared with HAP alone where cells were rounded	[252]
	GGGGGG GFHRRIK A		*In vitro* (hMSCs)	-↑ number of cells on HAP coated with FHRRIKA after 1h compared with HAP alone -Combination with RGD peptide had no effect on the number of cells -Cells slightly spread on HAP coated with	[252]

				FHRRIKA or combined with RGD after 1h compared with HAP alone where cells were rounded	

AdSC, adipose derived stem cells; BSA, bovine serum albumin

2.7.2 Homogeneous peptide-modified surfaces

One of the most frequent sequences in many proteins is the RGD peptide. A RGD sequence derived from the BSP (CGGNGEPRGDTYRAY, pRGD) grafted to PCL films induced the formation of focal adhesions and organization of the actin cytoskeleton in MC3T3-E1 preosteoblasts placed in serum-free medium for 1h. The cells also had a greater cell surface area than those on the negative peptide CGGNGEPRGETYRAY [57]. More importantly, only the MC3T3-E1 preosteoblasts attached to PCL functionalized by pRGD responded to BMP-2 (100 ng/mL). Shin *et al.* also used a composite scaffold of poly (L-lactide) (PLLA) and biphasic calcium phosphate (BCP) grafted with an RGD peptide to study the impact of adhesive peptides on the behavior of hMSCs [272]. The cells on the composite scaffold modified with the RGD peptide for 24h contained actin stress fibers and paxillin at the focal adhesions while those on the unmodified scaffold had none. There were also more spread cells on the RGD-modified scaffold. The hMSCs on the RGD-modified scaffold for 7 days had more ALP activity and, after 14 days, greater mineralization than did cells on the unmodified scaffold or PLLA grafted with RGD [272]. Since type I collagen is the major component of bone osteoid, several peptides derived from this protein such as DGEA and GFOGER have been developed [146,183,314]. The research group of Garcia has shown that GFOGER-coated PCL scaffolds significantly improved the repair of femoral bone defects in rats, 12 weeks after implantation [314]. Furthermore, Mehta *et al.* showed that the ALP activity of rat MSCs trapped in an alginate hydrogel containing a DGEA peptide (H$_2$N-GGGGDGEASP-OH) for 7 days was greater in comparison with the controls (alginate alone or in combination with RGD) [183]. Their production of OC and their mineralization were also increased (242).

Other peptides that could improve the adhesion, proliferation and differentiation of bone cells are those containing a heparin-binding site that interacts with heparan sulphate proteoglycans at the cell surface [51,285]. One peptide sequence that binds to heparan sulphate proteoglycans is Lys–Arg–Ser–Arg (KRSR), which is found in proteins such as fibronectin, vitronectin and BSP [53]. Sun *et al.* have shown that MC3T3-E1 preosteoblasts on titanium dioxide (TiO$_2$) anodized nanotubes functionalized with KRSR had more vinculin at focal points and a better organized actin cytoskeleton than did those on nanotubes functionalized with the negative peptide KSRR or the control nanotubes [285]. The osteogenic differentiation of MC3T3-E1 preosteoblasts attached to TiO$_2$ anodized nanotubes functionalized with KRSR was also better, as shown by the increased concentrations of mRNA encoding osteogenic markers (ALP, BSP, Runx2, OPN, Osx and OC) and ALP activity after 7 and 14 days.

There are many other challenges in the use of peptides-functionalized surfaces, such as the way they are immobilized on the surface to retain their bioactivity, their density and conformation [59]. Rezania and Healy compared the effect of peptides derived from BSP (CGGNGEPRGDTYRAY) immobilized on a quartz surface at densities from 0.01 to 3.8 pmol/cm² on the osteoblastic differentiation of rat calvaria osteoblast-like cells [239]. A density of at least 0.62 pmol/cm² of BSP peptides was necessary to increase the mineralization in rat calvaria osteoblast-like cells.

The conformation of the grafted peptide also influences cell behavior. The RGD peptide can be used in a linear or cyclic conformation. Cyclic RGD peptide is more stable than the linear peptide at physiological pH [19]. Kilian *et al.* also showed that the cyclic RGD (RGDfC where f is a phenylalanine residue with a D configuration) enhanced focal adhesion and increased MSC spreading more than did the linear RGD (GRGDSC) [138]. The cyclic RGD peptide could also enhance the affinity of cells for the alphav beta3 integrin better than did the linear one [12,221].

Another important challenge is the sterilization of biomimetic materials that use bioactive molecules. The main sterilization methods used are moist heat, dry heat, gamma radiation, ultraviolet radiation, hydrogen peroxide, and ethylene oxide [34,112]. RGD peptide dissolved in ultrapure water is completely broken down after exposure to UVC for 12h, while

ethylene oxide sterilization alters proteins by forming adducts between the ethylene oxide and the protein methionine and cysteine residues [34,112].

2.7.3 Mixed peptide surfaces

Combinations of adhesive peptides

It is possible to immobilize a combination of peptides derived from different ECM proteins on the biomaterial surface so as to mimic more precisely bone physiology and so improve the interaction between the material and the cells. The frequently used RGDS peptides have been immobilized together with KRSR peptides on silk fibroid nanofibers [139]. This combination increases the proliferation of human osteoblasts over that of cells on nanofibers of silk fibroid alone. However, the potential of RGD and KRSR peptides combinations for bone application may be controversial. A combination of GCRGYGRGDSPG and GCRGYGKRSRG peptides on a titanium implant has no greater synergistic effect on bone formation in maxillae of miniature pigs than does KRSR alone [23].

The two immobilized peptides can also be derived from the same protein. Fibronectin possesses a synergistic site PHSRN in its $FNIII_9$ domain that enhances the interaction between the RGD sequence in its $FNIII_{10}$ domain and alpha5 beta1 integrin [8]. Chen *et al.* developed a simple technique for immobilizing the bioactive fibronectin motifs, RGD (KKKGGGGRGDS) and PHSRN (KKKGGGGPHSRN) on Ti surfaces modified with (3-chloropropyl) triethoxysilane (CPTES) [36]. This combination of RGD and PHSRN peptides enhanced the adhesion of MC3T3-E1 preosteoblasts more than did either of the peptides alone, but there were no difference in the osteoblastic differentiation after 14 days [36]. The best proportion between the two peptides that favors the desired cell response and tissue repair must be determined. Nakaoka *et al.* used alginate gels functionalized with different ratios of RGD (GGGGRGDSP) and PHSRN (GGGGPHSRN) peptides to show that cells seeded on alginate containing 67 percent RGD and 33 percent PHSRN peptides had more OC than did cells on gels containing 11 percent RGD and 89 percent PHSRN [199]. The two bioactive RGD and PHSRN motifs can be combined to create a longer peptide and so overcome the peptide ratio problem. Benoit and Anseth developed a peptide containing the RGD and PHSRN motifs separated by 13 glycine residues ($RGDG_{13}PHSRN$) [13]. This peptide grafted on a poly

(ethylene) glycol (PEG) hydrogel favored the organization of the actin cytoskeleton in osteoblasts from neonatal rat calvaria more than did ungrafted PEG.

Peptides derived from the adhesive protein fibronectin can be immobilized with peptides extracted from type I collagen so as to better mimic the properties of the bone matrix. Reyes *et al.* immobilized the peptide GFOGER (GGYGGGPC(GPP)$_5$GFOGER(GPP)$_5$GPC) that targets the integrin alpha2 beta1 with a fragment of fibronectin containing the sequences RGD and PHSRN recognized by alpha5 beta1 integrins [79,235]. This strategy activates specific integrins and improves both the adhesion of human fibrosarcoma cells and the activation of their FAK more than any of the peptides alone. Visser *et al.* designed a peptide containing the decapeptide collagen-binding (CBD) motif derived from the von Willebrand factor with an RGD motif at the C-terminus (WREPSFMALSGRGDS) [307]. Absorbable collagen type I sponges (ACSs) functionalized with CBD-RGD increased the ALP activity of rat spinal bone marrow MSCs by day 10 and enhanced mineralization of the matrix on day 21. Moreover, *in vivo* experiments in rats showed that with the injection of ACS functionalized by CBD-RGD in combination with BMP-2 formed ectopic bone after 21 days compared with ACSs containing BMP-2 alone [307].

These strategies can also be used to improve growth factor efficiency. The CBD motif has been used to develop chimeric recombinant proteins such as CBD-TGF-beta fusion proteins that enhance the delivery of the growth factor to the cells [96].

Mixed adhesive peptides with BMPs or their derived peptides

Adhesive peptides can be used in combination with growth factors such as the BMPs to develop synthetic osteoinductive materials (Tableau 2.2). Shekaran *et al.* designed a PEG gel functionalized with the GFOGER peptides (GGYGGGP(GPP)$_5$GFOGER(GPP)$_5$GPC) using maleimide chemistry in combination with embeded BMP-2 (0.03, 0.06 and 0.3 microg per 1.5 microL of hydrogel) [267]. Implanting these hydrogels in defects in the radius bone of B6129SF2/J wild type male mice resulted in better bone reconstruction in the presence of BMP-2 (mineral density and bone volume) than did implants of hydrogel without BMP-2.

Tableau 2.2 Targeting specific integrins to improve cell response to growth factors

Integrins	Growth factor	Culture system	Behavior	References
Beta1	BMP-2	*In vitro* (MC3T3-E1)	-Blocking the beta1 integrin subunit ↓ the BMP-2 transcription and secretion induced by mechanical stimulation -BMP-2 induces MC3T3-E1 differentiation (↑ in Runx2, Osx and ALP mRNA at 12h)	[176]
	BMP-2	*In vitro* (MSCs)	-↑ beta1 synthesis on alumina surface functionalized with BMP-2 after 2 days compared with alumina alone	[275]
Alpha2 Beta1	BMP-2	*In vivo* (B6129SF2/J wild-type mice)	-Scaffold targeting alpha2 beta1 integrin loaded with BMP-2 permitted better bone healing than scaffold targeting the integrin alone	[267]
	BMP-2, BMP-7	*In vitro* (hMSCs from healthy and osteoporotic patient)	↓ in alpha2 integrin subunit was concomitant with a ↓ in cell migration induced by the BMPs compared with control	[94]
Alpha5 Beta1	BMP-2	*In vitro* and *In vivo* (MSCs, Sprague-Dawley rats)	Fibronectin derived fragments: -↑ cell adhesion over hyaluronic acid hydrogel alone -↑ bone formation *in vivo* than hyaluronic acid hydrogel alone	[141]
	VEGF-A (20 ng/mL)	*In vitro* (HUVEC)	-↑ in amounts of alpha5 and beta1 integrin subunits (2-4h) -↑ in COX-2 mRNA induced by VEGF-A	[184]
	IGF-2	*In vitro* (hMSCs)	-Silencing alpha5 and beta1 integrin subunits ↓ IGF-2 synthesis - Alpha5 beta1↑ IGF-2 signaling via FAK and PI3K pathways	[95]
Alphav Beta3	VEGF	*In vitro* (HUVEC)	-↑ beta3 integrin phosphorylation in the presence of VEGF	[175]

			-Used antibodies blocking alphav and beta3 integrin subunits to inhibit VEGFR-2 phosphorylation	
	FGF-1	*In vitro* (NIH3T3)	-FGF-1 bound directly to alphav beta3	[195]
	FGF-1	*In vitro* (NIH3T3, HUVEC)	-FGF-1 formed ternary complex with the integrin alphav beta3 and the FGFR needed to maintain Erk1/2 phosphorylation	[324]
	IGF-1 (200 ng/mL)	*In vitro* (OSE-3T3)	-IGF-1 promoted Cbfa1 activity using the PI3K pathway -Used an inhibitor of alphav beta3 occupancy to inhibit Cbfa1 activity induced by IGF-1	[50]
	BMP-2	*In vitro* (MC3T3-E1)	-rCYR61 influenced the differentiation of MC3T3-E1 preosteoblasts by BMP-2 via the alphav beta3 integrin -↑ in rCYR61 induced an ↑ in BMP-2 synthesis	[284]
	pBMP-9 (400 ng/mL)	*In vitro* (MC3T3-E1)	-pBMP-9 induced ↑ in alphav integrin subunit in cell membrane -pBMP-9 induced more ↑ in ALP activity after 24h than in unstimulated cells	[179]
	RGD+pBMP-2	*In vitro* (hMSCs)	-↑ mineralization of hBMSCs after 14 days more than pBMP-2 or RGD alone	[193]

pBMP-2, peptide-derived from BMP-2

Both adhesive peptides and BMPs can be covalently bound to surfaces or scaffolds. He *et al.* functionalized a poly(lactide-ethylene oxide fumarate) (PLEOF) hydrogel with GRGD peptides, one peptide derived from residues 162−168 of OPN and another derived from residues 73−92 of BMP-2 [99]. Each peptide was covalently bound to the PLEOF polymer using three types of chemistry. The GRGD peptide contained an acrylamide function which bound to the crosslinker N,N-methylenebis(acrylamide) during the formation of the covalent gel, the OPN used an oxime reaction involving the –O-NH$_2$ function on the OPN peptide with the aldehyde group from the hydrogel, while the BMP-2-azide derived peptide was attached via a click chemistry involving the propargyl function of the hydrogel. He *et al.* used bone

marrow MSCs isolated from young adult Wistar rats to show a greater increase in the ALP activity (14 days) and calcium content (28 days) in MSCs on this hydrogel than in cells seeded on hydrogels containing RGD alone or RGD plus BMP-2 [99]. The combination of the 3 peptides (RGD, BMP-2 and OPN) also improved the production of vasculogenic markers like PECAM-1 and VE-cadherin *in vitro* [99]. Osteogenic and vasculogenic differentiation could greatly enhance the process of bone repair, as good vascularization of the biomaterial delivers more oxygen and the nutrients necessary for the proper functioning of bone cells. However, the size of the protein (for example BMP) used in combination with short adhesive peptides can block the interaction between the cells and the short peptides.

BMPs can be replaced by short derived peptides to overcome this problem. These peptides can mimic the knuckle epitope of the BMPs and interact with type II receptors such as BMPRII [24]. Our research group has developed a peptide derived from BMP-9 (pBMP-9) based on the studies of Suzuki *et al.* and Saito *et al.* on peptides derived from the knuckle epitope of BMP-2. We have shown that pBMP-9 (Ac-CGGKVGKACCVPTKLSPISVLYK-NH2) promoted the differentiation of murine MC3T3-E1 preosteoblasts in the same way as the entire protein BMP-9 [16,247,287]. It also induced woven bone formation when injected, together with chitosan, into the quadriceps of mice [14]. However, the early differentiation of murine MC3T3-E1 preosteoblasts seems to depend on the type of integrins involved in cell adhesion. Marquis *et al.* found that murine MC3T3-E1 preosteoblasts in the presence of pBMP-9 had enhanced ALP activity when they were adhered to polystyrene coated with BSP-derived peptides, while cells on peptides DGEA-coated polystyrene targeting alpha2 beta1 integrins showed no such increase [179].

Selection of the adhesive peptides to combine with peptides derived from BMPs is therefore critical. He *et al.* immobilized the adhesive peptides GRGD (1.62 pmol/cm^2) and peptide P4 derived from BMP-2 (KIPKASSVPTELSAISTLYL) (5.2 pmol/cm^2) on PLEOF hydrogels [98]. This combination acted synergistically on the commitment of rat MSCs to osteoblast lineage by increasing the ALP activity and stimulating better mineralization of the matrix than did peptides grafted alone [98]. These same two peptides have been used to functionalize self-assembled monolayers using click chemistry, to create a concentration gradient [193]. The combination of equal amount of GRGDS and P4 (65 pmol/cm^2 of each

peptide) synergistically up-regulated the number of BSP transcripts in human bone marrow stromal cells and mineralization of their matrix without any osteogenic supplements [193]. Peptides derived from the knuckle epitope of BMP-2 (KIPKACCVPTELSAISMLYL), BMP-7 (TVPKPSSAPTQLNAISTLYF) and BMP-9 (KVGKASSVPTKLSPISILYK) and an adhesive peptide (GRGDSPC) have been grafted onto the surface of polyethylene terephthalate (PET) to investigate the differentiation of mouse MC3T3-E1 preosteoblasts [342]. This modified biomimetic surface increased the cell contents of Runx2 transcripts and the production of ECM. A recent study that evaluated the synergistic effect of a peptide derived from BMP-7 (GQGFSYPYKAVFSTQ) and a cyclic RGD peptide grafted to a quartz substrate on the behavior of MSCs found that these two peptides increased the ALP activity and the matrix mineralization [328].

Another recently developed strategy uses self-assembled peptides that mimic the matrix (for review see [244]). These peptides can have a beta sheet motif and even a coiled-coil motif to assemble alpha helices into ordered structures. The osteoblastic differentiation of C2C12 cells can be increased by peptides amphiphiles TSPHVPYGGGS that bind with high affinity to BMP-2 [157].

We therefore should investigate combinations of several BMPs and growth factors with adhesive peptides so as to develop osteoinductive biomimetic materials that act in synergy to favor bone healing. This will be challenging because growth factors can trigger antagonistic intracellular signaling [180]. BMP-2, BMP-7 and BMP-9 are not members of the same BMP subfamily and they interact with different type I receptors. They therefore activate different non-canonical pathways. Our research group has recently demonstrated that BMP-2 and BMP-9 did not act synergistically when used to treat MC3T3-E1 preosteoblasts seeded on PCL films functionalized with a peptide derived from BSP due to different level of activation of the canonical Wnt pathway [58].

2.8 Conclusion

Bone healing process involves complex interactions between several cell types and signaling molecules. Autografts, the current gold standard for repairing bone defects, have several limitations. Therefore, biomaterials functionalized with adhesive peptides that favor

bone cell attachment have been combined with growth factors, especially BMPs and their derived peptides, in order to optimize bone healing. However, it is essential to understand the crosstalk between the integrins, which interact with these adhesive peptides, and growth factors so as to understand the signaling that will direct cell behavior such as the ability of stem cells to differentiate into mature osteoblasts. While it is true that using combinations of adhesive peptides and BMPs or their derived peptides to create biomimetic materials has given promising results, challenges remain, especially questions of peptide density, conformation, graft stability and sterilization methods.

2.9 Perspectives

We still face numerous challenges as we strive to create osteoinductive biomimetic materials in 3D using bioactive molecules since most current studies are performed in 2D cultures. Moreover, most studies have focused on the impact of BMPs on bone cell responses despite the fact that many other growth factors, such as FGF and VEGF, are involved in bone healing. We need a combination of several growth factors that together optimize the action of bone substitutes. Finding such a combination will not be easy, but will promise great benefits for the patients.

2.10 Acknowledgements

CHAPITRE 3.   EFFET DU PCL FONCTIONNALISÉ SUR LA RÉPONSE DE CELLULES SOUCHES MÉSENCHYMATEUSES EN PRÉSENCE DE PEPTIDES DÉRIVÉS DE LA BMP-9

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Contributions au document :

Cet article contribue au mémoire en présentant :

- L'organisation du cytosquelette d'actine et des points focaux et la détermination des sous-unités des intégrines présentes chez les MSCs C3H10T1/2 adhérant à des films de PCL-pBSP et de PCL-pFibro.
- L'activation de la FAK chez les MSCs C3H10T1/2 adhérant à des films de PCL-pBSP et de PCL-pFibro.
- L'activation de la voie des MAPK chez les MSCs C3H10T1/2 adhérant à des films de PCL-pBSP et de PCL-pFibro.
- L'effet d'une stimulation par la BMP-9 et par ses peptides dérivés sur la réponse des MSCs C3H10T1/2 adhérant à des films de PCL-pBSP et de PCL-pFibro en termes d'activation de la voie des Smad et leur translocation au noyau et d'activation de la voie des MAPK.
- L'effet d'un inhibiteur de JNK sur la capacité des MSCs C3H10T1/2 adhérant à des films de PCL-pBSP et de PCL-pFibro à exprimer Runx2 en présence de la BMP-9 et de ses peptides dérivés.

3.1 Résumé français

Les matériaux biomimétiques ont été développés afin de réguler le comportement des cellules souches. Nous avons analysé l'influence de films de polycaprolactone (PCL) fonctionnalisés par des peptides d'adhésion dérivés de la fibronectine (pFibro) ou de la sialoprotéine osseuse (pBSP) sur la réponse de cellules murines multipotentes C3H10T1/2 à la protéine morphogénétique osseuse-9 (BMP-9) et ses peptides dérivés (pBMP-9 et SpBMP-9). Le PCL-pFibro permet une meilleure organisation du cytosquelette et une activation plus rapide de la FAK que le PCL-pBSP. Le PCL-pFibro promeut une signalisation MAPK permettant d'améliorer la réponse des cellules à la BMP-9 en inactivant ERK1/2 et activant p38 et JNK. La BMP-9, le pBMP-9 et le SpBMP-9 induisent une phosphorylation et une translocation au noyau des Smad1/5/8 plus importantes chez les cellules incubées sur le PCL-pFibro par rapport à celles incubées sur le PCL-pBSP. La BMP-9 et ses peptides dérivés ont

restauré la phosphorylation de JNK chez les cellules incubées sur le PCL-pBSP, mais il demeure moins phosphorylé que chez les cellules incubées sur le PCL-pFibro en présence de pBMP-9 et SpBMP-9. Les cellules incubées sur le PCL-pFibro contiennent plus de Runx2, essentiel pour l'orientation des cellules souches vers les ostéoblastes, que les cellules sur PCL-pBSP lorsque stimulées par la BMP-9 et ses peptides dérivés. Runx2 n'est plus détecté lorsque les cellules sont prétraitées avec un inhibiteur de JNK. Ainsi, le pFibro en combinaison avec la BMP-9 et ses peptides dérivés semble être une stratégie prometteuse dans le développement de matériaux biomimétiques.

3.2 Graphical abstract
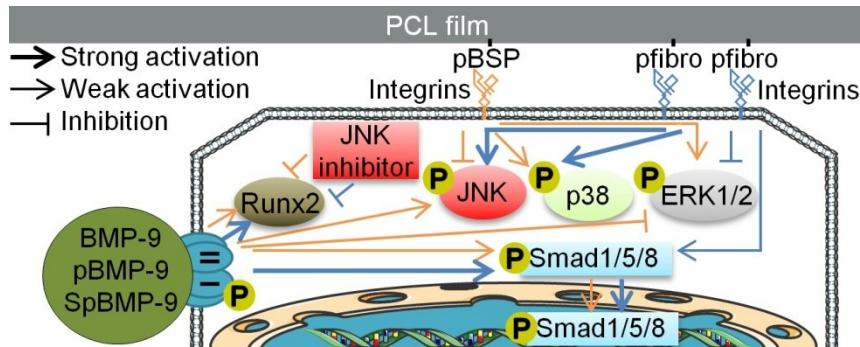
Figure 3.1 Graphical abstract **[**Illustration using Servier Medical Art, http://www.servier.fr]

3.3 Abstract

Biomimetic materials were developed to regulate stem cell behaviour. We have analysed the influence of polycaprolactone (PCL) films, functionalized with adhesive peptides derived from fibronectin (pFibro) or bone sialoprotein (pBSP), on the response of murine multipotent C3H10T1/2 cells to bone morphogenetic protein-9 (BMP-9) and its derived peptides (pBMP-9 and SpBMP-9). PCL-pFibro promoted better cell cytoskeleton organisation and faster focal adhesion kinase activation than did PCL-pBSP. PCL-pFibro also promoted MAPK signalling to improve the cell response to BMP-9 by inactivating ERK1/2 and stimulating p38 and JNK. BMP-9, pBMP-9 and SpBMP-9 induced greater phosphorylation of Smad1/5/8 and their translocation to the nucleus in cells attached to PCL-pFibro than in cells on PCL-pBSP. BMP-9 and its derived peptides restored the phosphorylation of JNK in cells

on PCL-pBSP, but it remained less phosphorylated than in cells on PCL-pFibro stimulated with pBMP-9 and SpBMP-9. Cells attached to PCL-pFibro contained more Runx2, essential for stem cell commitment to become osteoblasts, than did cells on PCL-pBSP when incubated with BMP-9 and its derived peptides. Runx2 was no more detected when the cells were pre-treated with JNK inhibitor. Therefore pFibro plus BMP-9 and its derived peptides seem to be a promising strategy to develop biomimetic materials.

3.4 Introduction

Biomimetic materials that promote bone cell adhesion, survival and differentiation have generated a great interest over the past decade for the development of new bone substitutes and tissue engineering strategies [73,179,237,239,267]. For example, polycaprolactone (PCL) functionalized with adhesive peptides that mimic extracellular matrix proteins favours the adhesion of bone marrow stromal cells or preosteoblasts to its surface [57,334]. The most commonly used adhesive peptides contain the cell binding motif Arg-Gly-Asp (RGD), which is found in proteins like bone sialoprotein (BSP), vitronectin and fibronectin [179,224,239]. The RGD motif is recognized by several cellular heterodimeric $\alpha\beta$ transmembrane receptors, including the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [118,197]. In addition, the binding of $\alpha_5\beta_1$ integrins to the RGD motif of the fibronectin FIII-10 domain can be modulated by a PHSRN motif located in the FIII-9 module [8,134].

However, the effect of materials functionalized by adhesive peptides on the intracellular signalling and their impact on the subsequent cell response to growth factors like bone morphogenetic proteins (BMP) remain poorly understood [58,267]. BMPs, which are cytokines of the transforming growth factor-β family, act on cells by forming a tetrameric complex with two type I and two type II serine/threonine kinase receptors. This complex activates the phosphorylation of type I receptors by type II receptors, leading to stimulation of the intracellular Smad1/5/8 cascade (also called the canonical pathway). BMP binding to the receptor can also activate a pathway involving TGF-β1-activated tyrosine kinase 1 (TAK1) and the mitogen-activated protein kinase (MAPK)-like ERK1/2, p38 and JNK pathways [325]. 20 BMPs have been identified to date. Only recombinant human BMP-2 and BMP-7 are presently approved by the Food and Drug Administration for commercial use [28,313]. Nevertheless, BMP-9 had a stronger osteoinductive potential than BMP-2 [132].

However these BMPs are expensive to produce. Several teams have therefore developed less costly peptides that contain the sequence recognized by the type II BMP receptor [38,247,287]. This sequence, called the knuckle epitope, differs slightly in BMPs like BMP-2, BMP-7 and BMP-9 [24]. We therefore developed two peptides derived from BMP-9 (pBMP-9 and SpBMP-9) [14,15] based on the studies of Suzuki *et al.* [287] and Saito *et al.* [247] on peptides derived from the knuckle epitope of BMP-2. Here, we studied the effect of PCL functionalized with peptide derived from BSP (pBSP) or peptide containing fibronectin RGD and PHSRN motifs (pFibro) on the organization of focal adhesions and the activation of focal adhesion kinase (FAK) and MAPK proteins in murine multipotent C3H10T1/2 cells. We then verified the impact of this signalling on the ability of C3H10T1/2 cells to respond to BMP-9 and its derived peptides, pBMP-9 and SpBMP-9.

3.5 Materials and methods

3.5.1 Materials

The peptide pBSP (Ac-CGGNGEPRGDTYRAY-NH$_2$) derived from bone sialoprotein was synthesized by Celtek Peptides (Celtek Bioscience, TN, USA) and the peptide pFibro (Ac-CGGPHSRNGGGGGGRGDG-NH$_2$) derived from fibronectin motifs was synthesized by EZBiolab (Carmel, IN, USA), both with a final purity of 98%. Recombinant carrier-free human BMP-9, synthesized in Chinese Hamster Ovary cells, was purchased from R&D Systems (Minneapolis, MN, USA), while pBMP-9 and SpBMP-9 were synthesized by Celtek Peptides (Celtek Bioscience, TN, USA). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). 2-(2-pyridinyldithio) ethaneamine hydrochloride (PDEA) was purchased from GE Healthcare (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Primary rabbit antibodies against phosphorylated FAK (Y^{397} or Y$^{576/577}$), phosphorylated Smad1 (Ser$^{463/465}$)/Smad5 (Ser$^{463/465}$)/Smad8 (Ser$^{426/428}$), phosphorylated Smad1 (Ser$^{463/465}$)/Smad5 (Ser$^{463/465}$), total Smad1/5/8, total ERK1/2, total p38, phosphorylated ERK1/2 and phosphorylated p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary murine antibodies against phosphorylated JNK and primary rabbit antibodies against integrin subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary rabbit antibodies for immunostaining of phosphorylated FAK (pFAK, Y^{397}), vinculin

and FITC-conjugated anti-rabbit IgG secondary antibodies were purchased from Sigma (St. Louis, MO, USA). Peroxidase-conjugated anti-rabbit IgG secondary antibodies were purchased from GE Healthcare. JNK inhibitor SP600125 was purchased from Millipore EMD (Billerica, MA, USA).

3.6 Methods

3.6.1 Preparation of PCL films

PCL films were prepared as previously described [57]. They were washed with distilled water and covered with a solution of EDC/NHS (0.149 mg/mL) for 20 min. These films were washed with phosphate-buffered saline (PBS), and covered with PDEA for 1h. The films were washed with PBS once more, and covered with pBSP or pFibro (0.123 nmol/cm^2) for 30 min at room temperature with agitation.

3.6.2 Cell experiments

Cell culture

Murine multipotent C3H10T1/2 cells (clone 8; CCL-226, ATCC, Manassas, VA, USA) were used between passages 10 and 15. Cells were grown at 37°C under a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) without ascorbic acid, supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Wisent, Saint-Bruno, QC, Canada), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco®, Grand Island, NY, USA). Cells were detached by incubation with trypsin (Gibco®, Grand Island, NY, USA) for 5 min at 37°C and the trypsin was neutralized with trypsin inhibitor (Gibco®, Grand Island, NY, USA). The cells were collected by centrifugation, suspended in DMEM without FBS, and seeded on sterile PCL-pBSP and PCL-pFibro film.

Staining of actin stress fibres, DNA and immunolabelling of vinculin and pFAK

Cells were seeded at 10 000 cells/cm^2 on sterile PCL films and incubated at 37°C under a humidified 5% CO_2 atmosphere for 4h in FBS-free medium. They were then fixed with 3% (w/v) paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 5 min at room temperature. Non-specific binding sites were blocked by incubating the PCL films with 3% (w/v) bovine serum albumin (BSA)

in PBS or 3% (w/v) skim milk for 45 min at 37°C under a humidified 5% CO_2 atmosphere. Cells were then immunostained by incubation for 30 min at 37°C under a humidified 5% CO_2 atmosphere with rabbit monoclonal anti-vinculin antibodies (diluted 1/150) or rabbit primary antibodies against phosphorylated FAK (pFAK) on Y^{397} (diluted 1/100) and washed with PBS. The cells were incubated for 30 min at 37°C under a humidified 5% CO_2 atmosphere with FITC-conjugated anti-rabbit immunoglobulins (diluted 1/400 to stain vinculin and diluted 1/300 to stain pFAK) containing also Alexa Fluor® 594 phalloidin (1/100, Invitrogen, Eugene, OR, USA) to stain filamentous actin (F-actin) and 4-6-diamidino-2-phenylindole (DAPI, Sigma) (1µg/mL) to stain nucleus. The surfaces were mounted on glass microscope slides and examined with an Eclipse TE2000-S microscope equipped with a 60x objective and a Retiga 1300R camera.

Western blot analysis of integrin subunits, FAK and MAPK phosphorylation

Cells were seeded at 20 000 cell/cm² on sterile PCL films and incubated at 37°C under a humidified 5% CO_2 atmosphere for 0, 0.25, 0.5, 1, 2 and 4h in FBS-free medium. They were then washed with PBS and lysed with 50 mM Tris-HCl containing 0.1% (v/v) SDS, a complete mini-protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and 1 mM orthovanadate. Equal amounts of protein were separated by SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Mississauga, ON, Canada) and the membranes were washed with 0.1% (v/v) Tween 20 in PBS. The western blots were performed as previously described [58,179]. Densitometric analyses were done using a spot densitometry image analysis program (Matlab).

Western blot analysis of Smad1/5/8 or MAPK induced by BMP-9 and its derived peptides

Cells seeded at 20 000 cell/cm² on sterile PCL films were incubated at 37°C under a humidified 5% CO_2 atmosphere for 4h in FBS-free medium. They were then stimulated by incubation with 1 nM of BMP-9, pBMP-9 or SpBMP-9 in 1% (v/v) FBS for 0.25, 0.5, 1, 2, and 4h. Western blots were done as previously described [58].

Immunolabeling of phosphorylated Smad1/5

Cells seeded at 20 000 cell/cm² on sterile PCL films were incubated at 37°C under a humidified 5% CO_2 atmosphere for 4h in FBS-free medium. They were then stimulated by incubation with 1 nM of BMP-9, pBMP-9 or SpBMP-9 in 1% (v/v) FBS for 2h. The cells were fixed, permeabilized and then incubated with 3% (w/v) BSA in PBS for 45 min at 37°C to block non-specific binding sites and immunostained by incubation for 30 min with rabbit monoclonal anti-phosphorylated Smad1/5 (diluted 1/50). They were incubated for 30 min with FITC-conjugated anti-rabbit IgG (diluted 1/100) containing DAPI (1µg/mL) to label the nucleus. The films were mounted as described above.

Western blot analysis of Runx2 with or without JNK inhibitor

Cells (20 000 cells/cm² on sterile PCL-pFibro or PCL-pBSP films) were incubated for 3h in FBS-free medium and then for 1h with 20 µM of JNK inhibitor SP600125. They were then stimulated with 1 nM of BMP-9, pBMP-9 or SpBMP-9 plus 1% (v/v) FBS for 3 days to determine Runx2 protein levels by western blots as described above.

3.6.3 Statistical analysis

Analysis of variance (ANOVA) and subsequent statistical analyses (Tukey-Kramer studentized range post-hoc tests) were performed using Excel (Excel 2010®). Only differences with a $p<0.05$ were considered significant.

3.7 Results

3.7.1 Effect of PCL functionalized with pBSP or pFibro on cytoskeleton organization and integrin subunits in murine multipotent C3H10T1/2 cells

We first investigated the ability of murine multipotent C3H10T1/2 cells seeded on PCL films functionalized with pBSP (PCL-pBSP) or pFibro (PCL-pFibro) for 4h in serum-free medium to organize their focal adhesions and actin cytoskeleton (Figure 3.2A). Hydrolysed PCL (PCL-Hydro) was used as a negative control. The C3H10T1/2 cells on PCL-Hydro spread poorly and displayed neither focal adhesion points nor organized actin cytoskeleton. In contrast, the cells attached to PCL-pBSP had focal complexes and thin focal adhesion points at their periphery. However, they contained few actin stress fibres. On PCL-pFibro, cells had

well developed focal adhesions and actin stress fibres. Therefore, the murine multipotent C3H10T1/2 cells seem to interact better with the PCL-pFibro than with PCL-pBSP. This might be due to the availability of integrin subunits in C3H10T1/2 cells, since pFibro binds mainly to β_1 integrin subunits [134], while pBSP interacts with $\alpha_v\beta_3$ integrins [179].
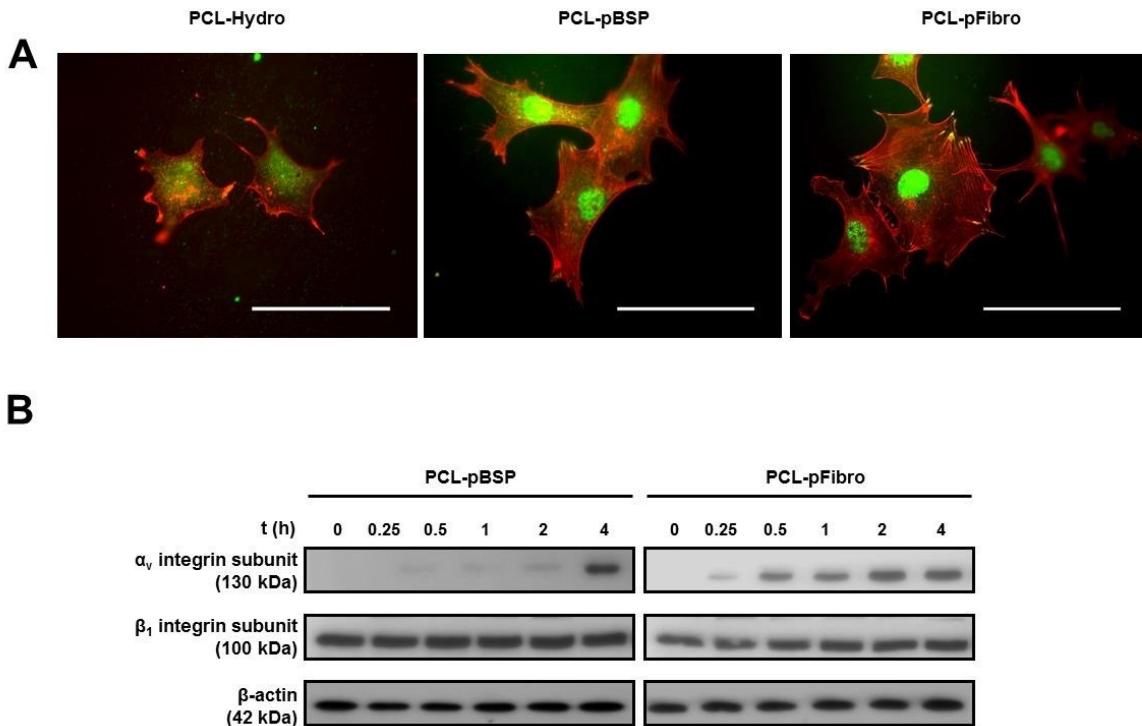
Figure 3.2 (A) C3H10T1/2 cells on PCL-Hydro, PCL-pBSP or PCL-pFibro were incubated for 4h in FBS-free medium. Attached cells were fixed, permeabilized and stained with Alexa Fluor® 594 phalloidin to label filamentous actin (F-actin, red) and immunolabelled for vinculin (green) Bar=100 µm. (B) Western blot analyses of β_1 and α_v integrin subunits in lysates of C3H10T1/2 cells attached to PCL-pBSP or PCL-pFibro for 4h in FBS-free medium. This experiment is representative of 2 independent experiments.

The amount of α_v and β_1 integrin subunits in cells attached to PCL functionalized with adhesive peptides was then determined after 0.25, 0.5, 1, 2 and 4h of incubation in serum-free medium (Figure 3.2B). Western blot analyses revealed that cells attached to PCL-pBSP contained a large amount of β_1 integrin subunits that remained stable for 4h. In contrast, these cells on PCL-pBSP contained very little α_v integrin subunits and this was only detected after 0.5h of incubation. The amount of α_v integrin subunit was much greater at 4h. The C3H10T1/2 cells attached to PCL-pFibro also contained a large amount of β_1 integrin subunits that

remained stable for 4h. These cells also contained more α_v integrin subunits than did cells attached to PCL-pBSP for 0.5, 1 and 2h.

3.7.2 Effect of PCL functionalized with pBSP or pFibro on FAK activation

Focal adhesion formation and turnover are regulated by the phosphorylated state of the FAK and changes in its conformation [74]. FAK activation first requires its autophosphorylation on Y^{397}, enabling it to be recognized by Src family tyrosine kinases [255]. Src then increases the activity of FAK by phosphorylating it on its catalytic site at $Y^{576/577}$ [74]. We confirmed the better interaction of C3H10T1/2 cells with PCL-pFibro in contrast to PCL-pBSP in serum-free medium by determining the rates at which FAK was activated on Y^{397} or $Y^{576/577}$ after incubation for 0, 0.25, 0.5, 1, 2 and 4h (Figure 3.3A and B). Cells attached to PCL-Hydro contained no pFAK within 4h. In contrast, cells attached to PCL-pBSP contained Y^{397} pFAK after 1h which increased between 1h and 4h. $Y^{576/577}$ pFAK was detected in cells attached to PCL-pBSP after incubation for 2h. Cells attached to PCL-pFibro contained Y^{397} pFAK at 0.5h and $Y^{576/577}$ pFAK after incubation for 1h. The band corresponding to Y^{397} pFAK (1h incubation) from cells on PCL-pFibro was more intense than the corresponding band from cells on PCL-pBSP. These observations were confirmed by densitometric analyses of bands corresponding to Y^{397} pFAK and referred to that of β-actin (Figure 3.3B). The amount of Y^{397} pFAK in cells attached to PCL-pFibro for 1h was 2-fold greater than that of cells on PCL-pBSP. However, the amounts of Y^{397} pFAK in cells on both adhesive peptides for 4h were quite similar. Immunolabelling of Y^{397} pFAK also showed that the kinase was located at the cell periphery in structures with the characteristics of focal adhesion points in cells attached to either PCL-pBSP or PCL-pFibro for 4h (Figure 3.3C). However, other signalling pathways, especially MAPK, can be activated during the attachment of cells to extracellular matrix proteins [130].
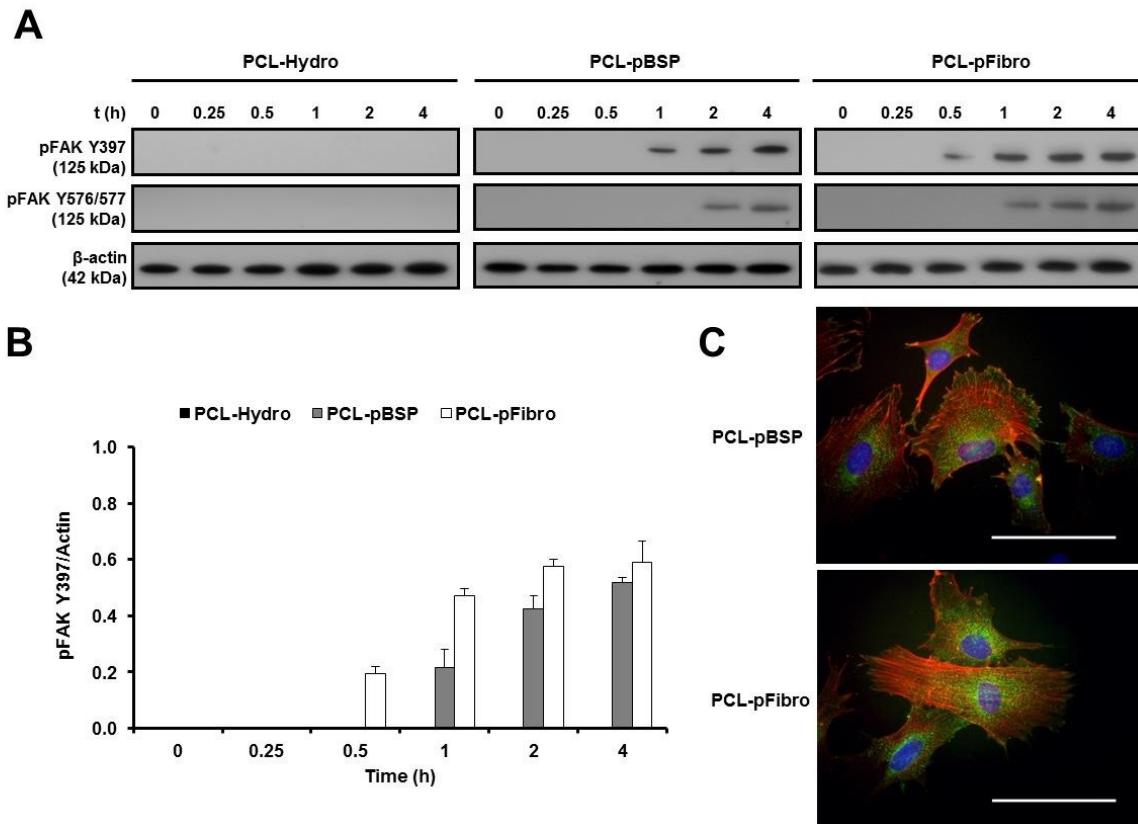
Figure 3.3 Kinetics of phosphorylated FAK Y^{397} and FAK $Y^{576/577}$ and β-actin amount determined by (A) western blotting and (B) densitometry. C3H10T1/2 cells on PCL-Hydro, PCL-pBSP or PCL-pFibro were incubated for 0.25, 0.5, 1, 2, and 4h in FBS-free medium. Error bars show standard errors of the mean (SEM) for three independent experiments. (C) C3H10T1/2 cells on PCL-pBSP or PCL-pFibro were incubated for 4h in FBS-free medium, fixed, permeabilized and incubated with Alexa Fluor® 594 phalloidin to label F-actin (red), DAPI to label nucleus (blue) and rabbit antibodies against Y^{397} pFAK (green). Bar = 100 μm. This experiment is representative of 2 independent experiments.

3.7.3 Effect of PCL functionalized with pBSP or pFibro on MAPK pathways

We analysed the phosphorylated states of p38, ERK1/2 and JNK in C3H10T1/2 cells attached to PCL-Hydro, PCL-pBSP or PCL-pFibro (for 0, 0.25, 0.5, 1, 2 and 4h) by western blotting (Figure 3.4A). The intensities of the bands corresponding to total p38, ERK1/2 proteins and β-actin (42 kDa), which were used as controls, were similar in all experimental conditions. Cells attached to PCL–Hydro contained no phosphorylated p38 (p-p38) or JNK (pJNK) within 4h. These cells gave only a very thin band corresponding to phosphorylated

ERK1/2 (pERK1/2) at 0.25h. In contrast, the amount of pERK1/2 in cells attached to PCL-pBSP was unchanged from 0.25h to 1h and then decreased to 4h. The rate of pERK1/2 activation in cells attached to PCL-pFibro was maximal between 0.5h and 1h. However, the pERK1/2 bands in cells on PCL-pFibro were less intense than those from cells on PCL-pBSP at 2h and 4h. p-p38 was detected in cells attached to PCL-pBSP for 4h, while the amount of p-p38 in cells on PCL-pFibro increased between 1h and 4h. Densitometric analyses of the pERK1/2 and p-p38 bands, with their respective total protein bands as references, confirmed the above observations (Figure 3.4B).
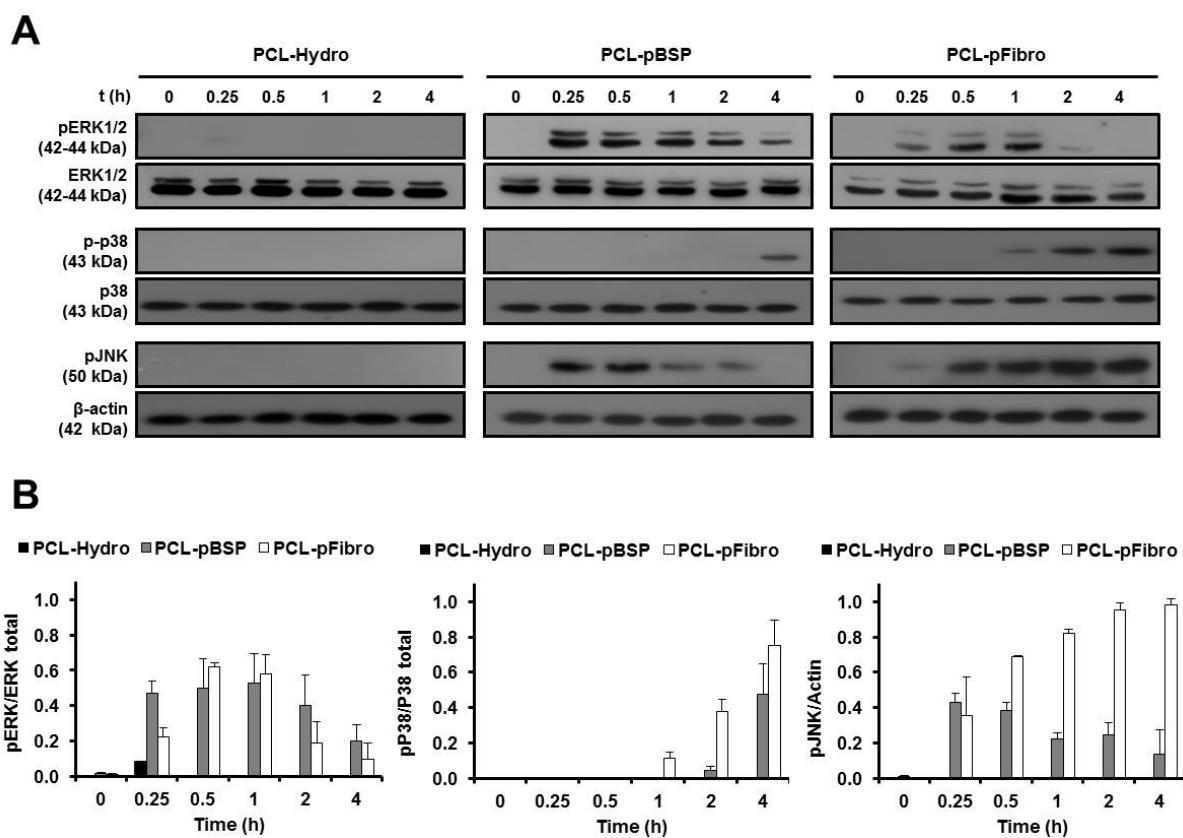
Figure 3.4 Kinetics of ERK1/2, p38 and JNK phosphorylation and total p38, ERK1/2 and β-actin determined by (A) western blotting and (B) densitometry. Error bars show SEM for two independent experiments. C3H10T1/2 cells were incubated on PCL-Hydro, PCL-pBSP or PCL-pFibro for 0.25, 0.5, 1, 2, and 4h in FBS-free medium. This experiment is representative of 3 independent experiments.

JNK activation reflects the major effect of the type of adhesive peptide on the MAPK pathway (Figure 3.4A). The intensity of the protein band corresponding to pJNK in cells

attached to PCL-pBSP decreased between 0.25h and 4h while this band increased in cells attached to PCL-pFibro (0.25h - 1h) and reached a plateau at 2h. Densitometric analyses of the pJNK bands referred to that of actin confirmed that at 2h, there was 4-fold less pJNK in cells attached to PCL-pBSP than in those on PCL-pFibro (Figure 3.4B). We have previously shown that the canonical Smad1/5/8 pathway in MC3T3-E1 preosteoblasts on a PCL-pRGD (or pBSP) is only activated by BMP-2 (50 ng/mL), while it is not in cells on film bearing pRGE negative peptides (PCL-pRGE) [57]. We, therefore, examined the influence of the type of adhesive peptides used to develop biomimetic materials on the response of murine multipotent cells to BMP-9 and its derived peptides.

3.7.4 Effect of PCL-pBSP and PCL-pFibro on the cell signalling induced by BMP-9 and its derived peptides

Smad 1/5/8 activation and their nuclear translocation

We used western blotting to measure the phosphorylated Smad1/5/8 (pSmad1/5/8) and thus determine the response of C3H10T1/2 cells attached to PCL-pBSP or PCL-pFibro to BMP-9 and its derived peptides (Figure 3.5A). The total Smad1/5/8 bands, used as control, were similar in all experiments. The C3H10T1/2 cells on PCL-Hydro and incubated for 0.5h to 4h with BMP-9, pBMP-9 or SpBMP-9 contained no pSmad1/5/8. Those on PCL-pBSP without BMP gave a very thin pSmad1/5/8 band, but the intensity of pSmad1/5/8 band from cells on PCL-pFibro increased over time. Cells on PCL-pBSP or PCL-pFibro treated with BMP-9, pBMP-9 and SpBMP-9 gave more intense pSmad1/5/8 bands. In addition, the intensity of the band from cells on PCL-pFibro stimulated with BMP-9 or its derived peptides seemed to be greater than those from stimulated cells on PCL-pBSP. However, the phosphorylated bands in cells on PCL-pFibro were more intense at 0.25h when incubated with BMP-9 than those of cells incubated with pBMP-9 or SpBMP-9. Nonetheless, the intensity of pSmad1/5/8 bands in cells attached to PCL-pFibro with BMP-9 or its derived peptide between 0.5h and 4h was quite similar.
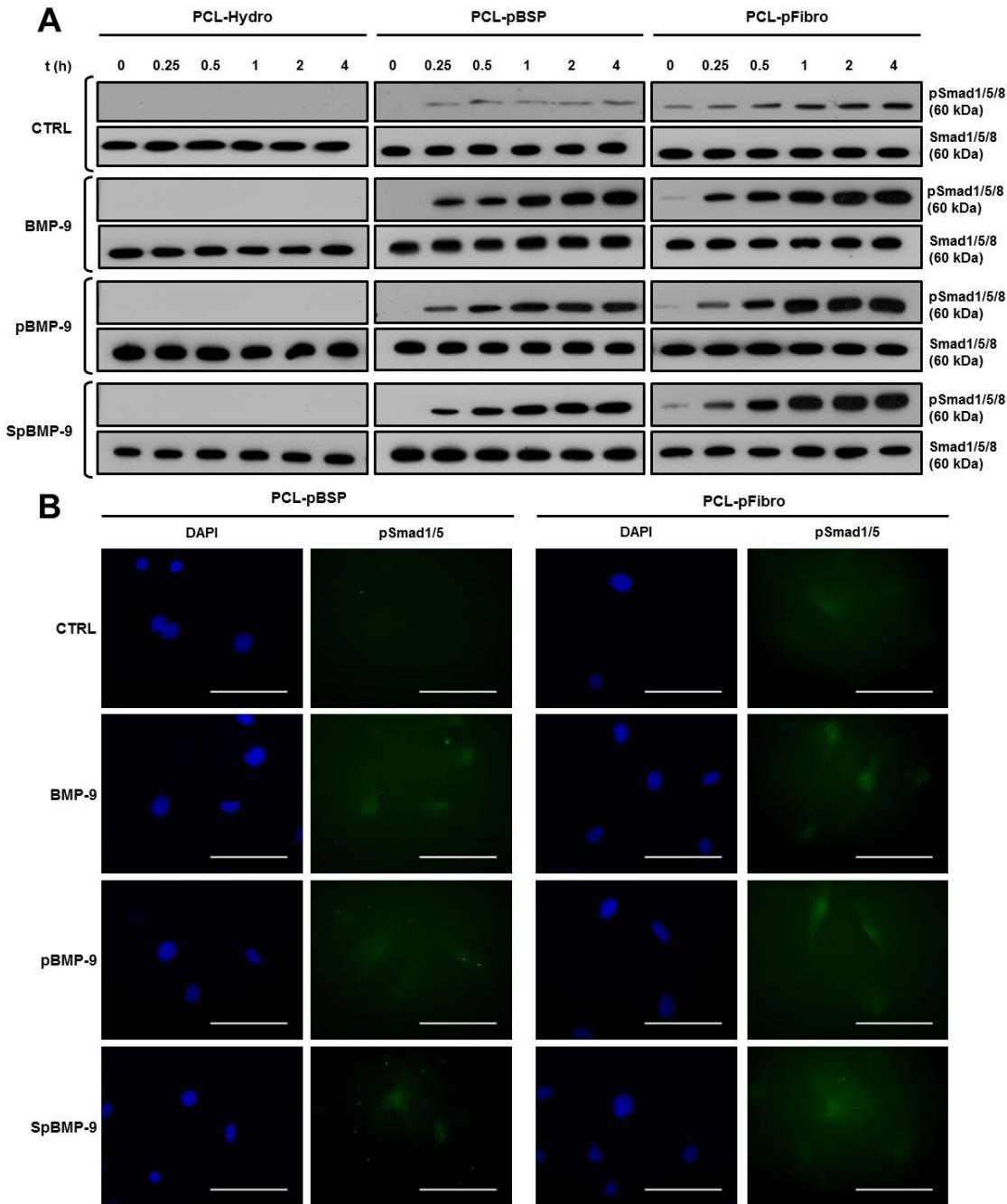
Figure 3.5 Kinetics of Smad1/5/8 phosphorylation and total Smad1/5/8 amount determined by western blotting. The C3H10T1/2 cells were seeded on PCL-Hydro, PCL-pBSP or PCL-pFibro for 4h in FBS-free medium and then stimulated with 1 nM BMP-9, pBMP-9 or SpBMP-9 plus 1% (v/v) FBS for 0.25, 0.5, 1, 2, and 4h. This experiment is representative of 2 independent experiments. (B) Translocation of pSmad1/5, visualised by immunolabelling (green), in cells attached at 4h to PCL-pBSP or PCL-pFibro in FBS-free medium and then stimulated with 1 nM BMP-9, pBMP-9 or SpBMP-9 for 2h in presence of 1% (v/v) FBS. The nuclei were labelled by DAPI (blue). Bar = 100 μm. This experiment is representative of 2 independent experiments.

We used immunostaining to verify the translocation of pSmad1/5 to the nucleus since it is required for the activation of the genes encoding osteogenic markers like Runx2 [62,147]. DAPI staining of nucleus was also used to confirm the nuclear location of pSmad1/5 (Figure 3.5B). There was some immunostaining of pSmad1/5 in untreated cells that attached to PCL-pFibro, while fewer pSmad1/5 were detected in cells on PCL-pBSP. In contrast, C3H10T1/2 cells on both PCL-pBSP and PCL-pFibro that were treated with BMP-9 or its derived peptides contained more pSmad1/5 staining than the control. However, pBMP-9 and SpBMP-9 seemed to induce less intense staining for pSmad1/5 in cells on PCL-pBSP than in cells on PCL-pFibro. The pSmad1/5 in cells on PCL-pBSP and treated by BMP-9 were mainly located in the nuclei, while this nuclear translocation was incomplete in cells stimulated with pBMP-9 or SpBMP-9. Since the translocation of pSmad1/5/8 to the nucleus can be affected by ERK1/2 activation [147], we then assessed the MAPK pathway in cells on PCL-pBSP or PCL-pFibro and incubated with BMP-9 and its derived peptides.

MAPK activation

The C3H10T1/2 cells incubated for 4h on PCL-pBSP or PCL-pFibro in serum-free medium and then stimulated by 1 nM of BMP-9 or its derived peptides for 0.25, 0.5, 1, 2 or 4h plus 1% (v/v) FBS were subjected to western blotting for pERK1/2 (Figure 3.6A) and p-p38 (Figure 3.6B). The intensity of the bands corresponding to total p38, ERK1/2 proteins and β-actin (42 kDa), used as controls, were similar in all the experiments. The only unstimulated cells that contained pERK1/2 were those on PCL-pBSP. Stimulating the cells on PCL-pBSP with BMP-9 decreased their pERK1/2 content, so that it had disappeared by 0.25h. In contrast, the cells on PCL-pBSP that had been treated with pBMP-9 or SpBMP-9 contained pERK1/2 for up to 1h. The cells on PCL-pFibro produced no ERK1/2 band when incubated with or without BMP-9, pBMP-9 or SpBMP-9. The unstimulated cells on both functionalized PCL films produced p-p38 (Figure 3.6B). Moreover, incubation of cells on PCL-pFibro with SpBMP-9 for 2h and 4h increased their p-p38 content (Figure 3.6C).
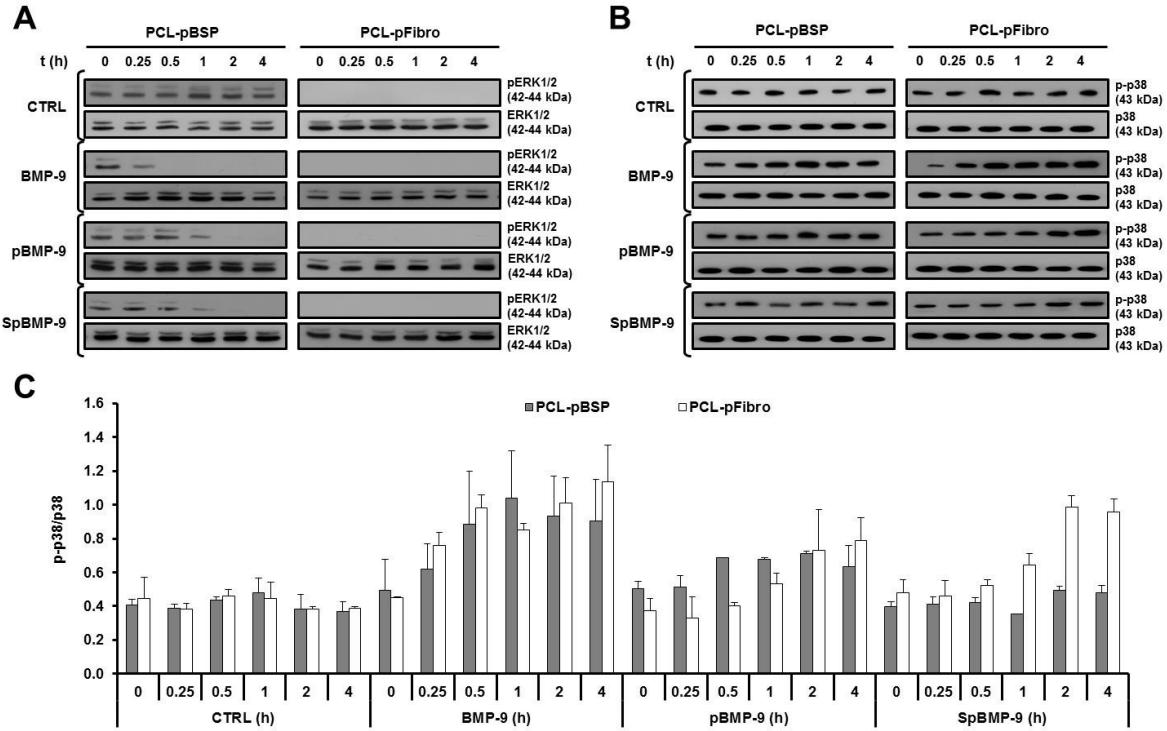
Figure 3.6 Kinetics of phosphorylated (A) ERK1/2 and (B) p38 determined by western blotting and (C) densitometry. Error bars show SEM for 2 independent experiments. The C3H10T1/2 cells were seeded on PCL-Hydro, PCL-pBSP or PCL-pFibro for 4h in FBS-free medium and then stimulated with 1 nM BMP-9, pBMP-9 or SpBMP-9 for 0.25, 0.5, 1, 2, or 4h plus 1% (v/v) FBS.

Western blotting for pJNK (Figure 3.7A) showed that the cells on PCL-pBSP contained no pJNK at 4h, while the pJNK amount in those on PCL-pFibro was high. BMP-9 or its derived peptides induced the phosphorylation of JNK after 0.25h in cells on PCL-pBSP and this amount increased between 0.25h and 4h as observed by densitometry (Figure 3.7B). BMP-9 or its derived peptides also slightly increase the pJNK concentration in cells on PCL-pFibro. The C3H10T1/2 cells on PCL-pFibro that had been treated with BMP-9 had a higher pJNK content (~1.8-fold) between 0h to 2h than the cells on PCL-pBSP ($p<0.001$ for 0h and $p<0.05$ for 2h). Furthermore, pBMP-9 or SpBMP-9 stimulated cells on PCL-pFibro contained 10-fold more pJNK at 0.25h than did cells on PCL-pBSP ($p<0.001$), but this difference decreased to 2-fold at 4h ($p<0.001$).
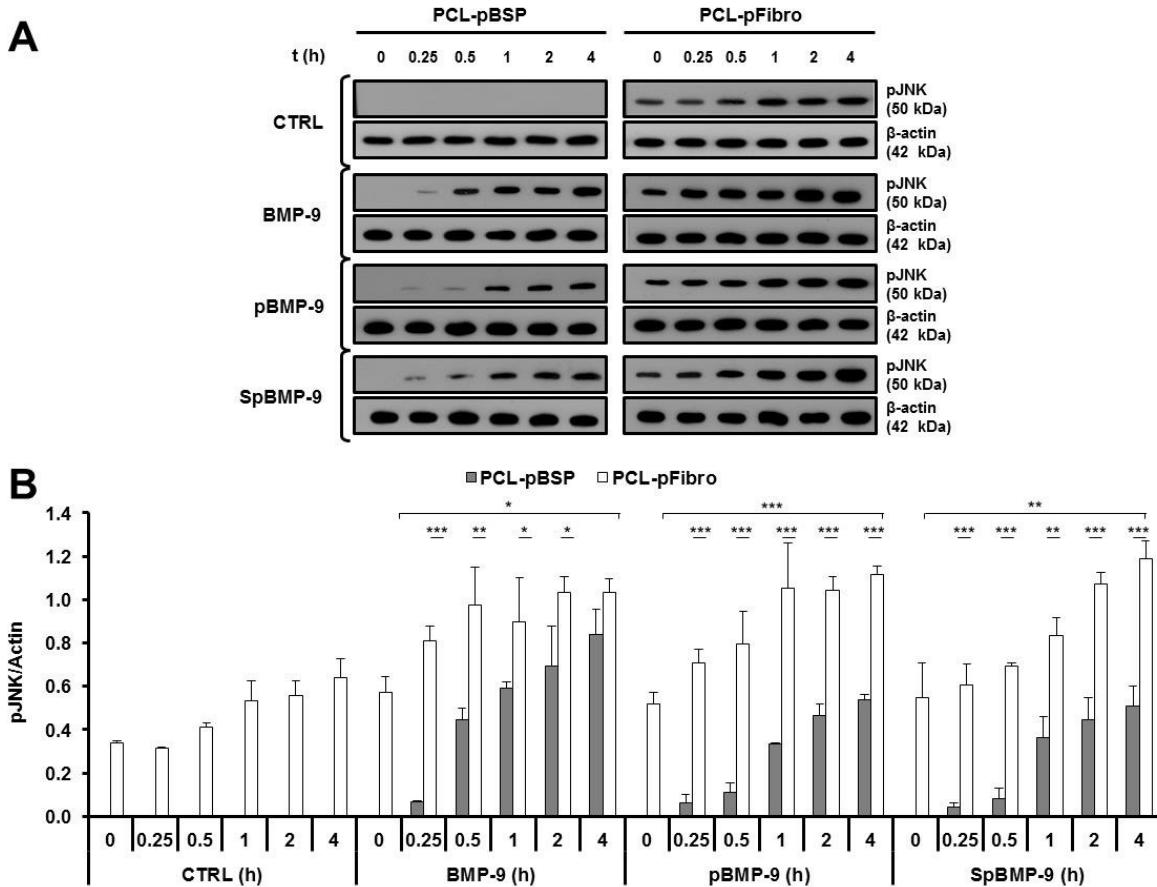
Figure 3.7 Kinetics of phosphorylated JNK was determined by (A) western blotting and (B) densitometry. Error bars show SEM for 2 independent experiments. The C3H10T1/2 cells were seeded on PCL-Hydro, PCL-pBSP or PCL-pFibro for 4h in FBS-free medium and then stimulated by incubation with 1 nM BMP-9, pBMP-9 or SpBMP-9 for 0.25, 0.5, 1, 2, or 4h in the presence of 1% (v/v) FBS. * p<0.05, ** p<0.01, *** p<0.001.

3.7.5 Effect of JNK on the osteoblastic commitment of C3H10T1/2 cell on PCL-pBSP or PCL-pFibro stimulated with BMP-9 and its derived peptides

We pre-treated C3H10T1/2 cells with 20 μM SP600125, a JNK inhibitor, to determine whether JNK was involved in their osteoblastic commitment. We first used western blotting to verify that the JNK inhibitor blocked the phosphorylation of JNK in cells on PCL-pBSP or PCL-pFibro with or without 1 nM BMP-9 or its derived peptides (Figure 3.8A). We then determined the effect of JNK inhibitor on the amount of the transcription factors involved in the commitment of multipotent stem cells to become osteoblasts (Runx2), chondroblasts

(Sox9) or adipocytes (PPARγ) (Figure 3.8A). No Sox9 or PPARγ was detected under any of the experimental conditions. Runx2 was detected only in cells attached to PCL-pBSP or PCL-pFibro when BMP-9 or its derived peptides were present. However, densitometry revealed that there was significantly more Runx2 in cells attached to PCL-pFibro than in cells on PCL-pBSP in the presence of BMP-9 (p<0.01), pBMP-9 (p<0.01) or SpBMP-9 (p<0.001) (Figure 3.8B). There was also significantly more Runx2 in cells attached to PCL-pBSP and PCL-pFibro that had been incubated with BMP-9 than in cells incubated with pBMP-9 (p<0.01) or SpBMP-9 (p<0.001 for PCL-pBSP, p<0.05 for PCL-pFibro) (Figure 3.8B).
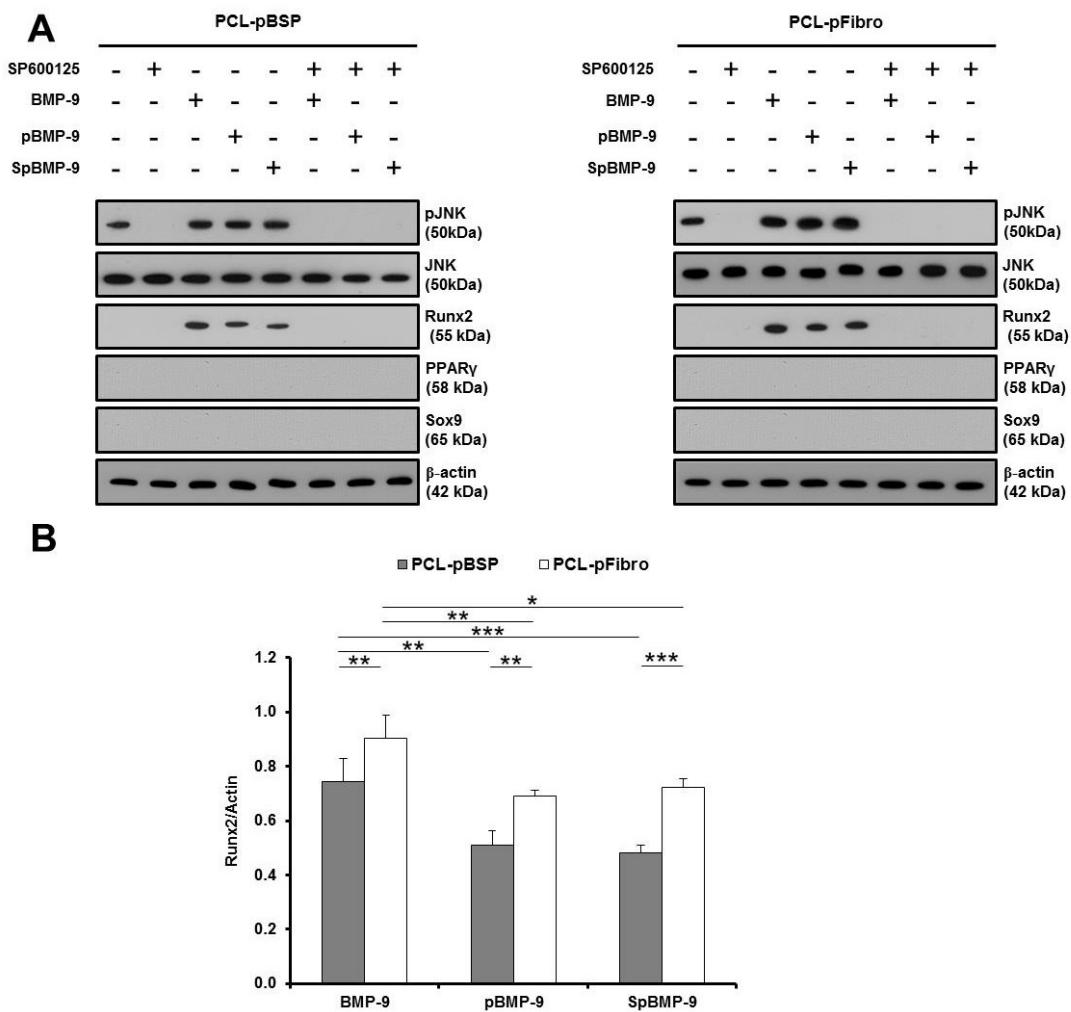
Figure 3.8 The effect of JNK inhibitor SP6001258 on pJNK, Runx2, PPARγ and Sox9 was studied using (A) western blotting and (B) densitometry. Error bars show SEM for 3 independent experiments. The C3H10T1/2 cells were seeded on PCL-pBSP and PCL-pFibro for 3h in FBS-free medium and then incubated for 1h with or without 20 μM SP600125 and then stimulated with 1 nM BMP-9, pBMP-9 or SpBMP-9 for 3 days in the presence of 1% (v/v) FBS. * p<0.05, ** p<0.01, *** p<0.001.

3.8 Discussion

Several studies have demonstrated that biomimetic materials functionalized with adhesive peptides targeting specific integrins can regulate the differentiation of bone cells [238,267]. These biomimetic materials also affect the ability of bone cells to respond to their environment such as growth factors but the mechanisms involved in such phenomena remain unclear [58]. We have now shown that two adhesive peptides, pBSP and pFibro, can modulate the response of stem cells to BMP-9 and its derived peptides by modulating cell attachment and associated signalling.

The adhesive peptides, pBSP and pFibro, grafted onto PCL films can both facilitate the attachment of C3H10T1/2 cells in serum-free condition. However, more focal adhesion points were formed and the actin cytoskeleton was better organized in C3H10T1/2 cells attached to PCL-pFibro than in the cells on PCL-pBSP. The small difference between the cells on PCL-pBSP and PCL-pFibro may be due to a difference in the availability of the α_v subunit, that interacts with pBSP, and the β_1 integrin subunit, that recognizes pFibro [134,179]. Moreover, the FAK activation induced by cells attachment to each adhesive peptide, at both its autophosphorylation site (Y^{397}) and its $Y^{576/577}$ site, agrees well with the focal adhesion site formation and cytoskeleton organization. Several studies on different substrates have shown that short adhesive peptides can activate FAK [222,334].

Furthermore, Tamura *et al.* [292] showed that FAK must be activated for preosteoblasts to respond to BMP-2. We found that FAK was activated in cells attached to either PCL-pBSP or PCL-pFibro, suggesting that these surfaces may favour BMP-9-mediated Smad1/5/8 activation. We detected pSmad1/5/8 in cells attached to PCL-pBSP and PCL-pFibro that had been incubated with BMP-9 or its derived peptides, while Smad1/5/8 remained inactive in cells on PCL-Hydro. Our results also indicate that there is more pSmad1/5/8 in cells attached to PCL-pFibro than in cells on PCL-pBSP, even without any BMP stimulation. Tian *et al.* [294] also observed that the Smad1/5/8 in human microvascular endothelial-1 cells (HMEC-1) attached to fibronectin-coated polystyrene were activated even without any BMP-9. Fibronectin also dose-dependently improves the phosphorylation of Smad1/5/8 induced by 2 ng/mL of BMP-9 at 30min in attached HMEC-1 [294]. The $\alpha_5\beta_1$ integrins, the BMP-9 type I receptor ALK-1 and the type III receptor endoglin that are found on endothelial cells are all

involved in this phenomenon [294]. Further experiments are now required to determine how pFibro promotes Smad1/5/8 activation. We also found that cells attached to PCL-pFibro had no pERK1/2 but contained large amounts of p-p38. Xu *et al.* [318] incubated C3H10T1/2 cells in conditioned medium taken from HCT116 infected with AdBMP-9, and found that inhibiting ERK1/2 increased Smad1/5/8 phosphorylation. We found that Smad1/5 is both better phosphorylated and translocated to the nuclei of C3H10T1/2 cells attached to PCL-pFibro and incubated with BMP-9 or its derived peptides than the Smad1/5 in cells on PCL-pBSP. BMP-9 was a better inducer of pSmad1/5 translocation to the nuclei of cells attached to PCL-pBSP than were pBMP-9 or SpBMP-9, perhaps because ERK1/2 remains phosphorylated on PCL-pBSP for at least 1h after adding pBMP-9 and SpBMP-9. Indeed, Kretzschmar *et al.* [147] found that ERK1/2 phosphorylates Smad1 at the linker region, thus inhibiting its translocation to the nucleus.

Furthermore, we found that JNK remained activated only in C3H10T1/2 cells attached to PCL-pFibro. Partially inhibiting the JNK in cells on PCL-pFibro also greatly decreased Smad1/5/8 phosphorylation, while increasing the pERK1/2 level and blocking p38 phosphorylation (Supplementary data 1). Liu *et al.* [165] found that inhibiting the JNK in MC3T3-E1 preosteoblasts treated with BMP-2 (100 ng/mL) blocks the phosphorylation of Smad1/5/8 by BMPR-I due to the interaction of this receptor with inhibitory Smad6.

Since PCL-pFibro seemed to favour both canonical and non-canonical BMP signalling, we examined its influence on the osteogenic commitment of murine multipotent C3H10T1/2 cells incubated with BMP-9, pBMP-9 or SpBMP-9 by assaying Runx2, Sox9 and PPARγ proteins. Runx2 is a key transcription factor that commits mesenchymal stem cells to the osteoblastic lineage, Sox9 is a marker of chondrogenic differentiation, and PPARγ promotes adipogenic differentiation [62,124,339]. Loebel *et al.* [168] recently demonstrated that the Runx2/Sox9 ratio can predict the ability of human mesenchymal stem cells extracted from human iliac crest bone and vertebral bodies to differentiate into mature osteoblasts, since down-regulation of Sox9 promotes the osteoblastic commitment. Our data on Runx2 and Sox9 protein amount suggest that multipotent C3H10T1/2 cells attached to PCL-pFibro and stimulated by BMP-9 or its derived peptides have a greater potential to differentiate into mature osteoblasts than do cells on PCL-pBSP. We also found that inhibiting JNK prevented

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BMP-9, pBMP-9 or SpBMP-9-induced protein expression of Runx2 by cells attached to functionalized PCL. Zhao *et al.* [340] found that 30 µM JNK inhibitor SP600125 decreased the ALP activity in C3H10T1/2 cells infected with AdBMP-9 and prevented matrix mineralization. These results demonstrate the importance of JNK in stem cell commitment to the osteoblastic lineage induced by pBMP-9 and SpBMP-9.

3.9 Conclusion

We found that PCL-pFibro is a better biomimetic material than PCL-pBSP for promoting the adhesion of murine multipotent cells and their response to BMP-9 and its derived peptides in terms of signalling and osteoblastic commitment. We have shown that JNK is an essential factor in the osteoblastic commitment of C3H10T1/2 induced by pBMP-9 and SpBMP-9. Nevertheless, further experiments are required to better understand the mechanism by which pBMP-9 and SpBMP-9 drive the faith of stem cells toward mature osteoblasts. PCL-pFibro combined with peptides derived from BMP-9 could lead to a strategy for treating bone defects.

3.10 Acknowledgments

CHAPITRE 4.   CONCLUSION ET PERSPECTIVES

4.1 Conclusion

Le vieillissement de la population aura comme effet d'augmenter le nombre de fractures ostéoporotiques menant à des fractures de taille critique, poussant ainsi le développement de nouvelles stratégies de comblement osseux afin de remédier aux problématiques occasionnées par les techniques actuelles de réparation. L'une de ces stratégies est l'utilisation de biomatériaux fonctionnalisés par des peptides d'adhésion en combinaison avec des facteurs de croissance.

Ce projet de maîtrise a d'abord permis d'étudier l'adhésion cellulaire de MSCs C3H10T1/2 sur des films de PCL fonctionnalisés par un peptide d'adhésion pBSP ou un peptide d'adhésion pFibro. Il a été démontré que le PCL-pFibro était favorable à l'adhésion des cellules et que cela était entre autres dû à la disponibilité des intégrines $\alpha_5\beta_1$ chez les C3H10T1/2. Cette adhésion plus propice sur PCL-pFibro se répercutait par la suite au niveau de l'activation des voies de signalisation favorable à la différenciation cellulaire. Les résultats ont aussi démontré que les 2 surfaces avaient un effet contraire sur l'activation de JNK, celui-ci étant essentiel à la différenciation des MSCs. Ces résultats montrent l'importance de sélectionner judicieusement les peptides d'adhésion servant à fonctionnaliser les surfaces en fonction des intégrines ciblées par ceux-ci.

Par la suite, l'effet de la BMP-9 et ses 2 peptides dérivés, le pBMP-9 et le SpBMP-9, sur les MSCs C3H10T1/2 ensemencées sur les surfaces de PCL fonctionnalisées par pBSP et pFibro a été testé. Les résultats ont permis d'observer que les 2 peptides dérivés de la BMP-9 permettaient de simuler l'effet de la protéine entière avec un léger retard. Il s'agit d'une contribution importante puisqu'il s'agit de la première fois que le pBMP-9 et le SpBMP-9 étaient utilisés pour stimuler des MSCs. Ces peptides pourraient donc être amenés à être utilisés en génie tissulaire en tant qu'alternative à la BMP-9 qui est coûteuse à produire.

En conclusion, ce projet de maîtrise a permis d'acquérir des connaissances sur la réponse des MSCs, d'abord face à des surfaces fonctionnalisées par des peptides d'adhésion et

par la suite face à une utilisation conjointe de surfaces fonctionnalisées et de BMP-9 et ses peptides dérivés. Bien que les cellules utilisées lors de cette étude ne soient pas d'origine humaine, les cellules d'origine murine C3H10T1/2 représentent un modèle couramment utilisé dans la littérature pour étudier la différenciation ostéogénique et ont permis d'obtenir des résultats concluants. Ces résultats constituent une avancée dans le développement de matériaux biomimétiques utilisés en tant que substituts osseux, mais de nombreux défis restent à relever.

4.2 Perspectives

4.2.1 Immunomarquage des intégrines

Dans le cadre de ce travail, une analyse des sous-unités des intégrines α_v et β_1 a été effectuée par immunobuvardage de type Western afin de vérifier leur expression. Par contre, un immunomarquage afin d'identifier leur localisation dans le temps au niveau des points focaux aurait été intéressant. Cet immunomarquage aurait permis d'identifier les intégrines impliquées au niveau de l'adhésion. En effet, Marquis *et al.* a démontré que les préostéoblastes murins MC3T3-E1 cultivés pendant 1h sur une surface de polystyrène recouverte de pBSP expriment la sous-unité β_1 au niveau de la membrane, mais la sous-unité β_1 n'est pas organisée au niveau des points focaux [179].

4.2.2 Dose-réponse

Lors de cette étude, les cellules ont été stimulées par une seule dose de BMP-9 ou de ses peptides dérivés, soit 1 nM. Cette concentration avait été choisie basée sur les résultats de Lauzon *et al.* qui a démontré qu'une dose de 1 nM de BMP-9 en présence de 10% (v/v) de FBS pendant 1h permettait d'obtenir une réponse maximale en termes d'activation de la voie des Smad1/5/8 chez les préostéoblastes MC3T3-E1 cultivés sur polystyrène [153]. Par contre, il est à noter que certaines conditions expérimentales diffèrent, notamment le type cellulaire, la concentration de FBS ainsi que la surface de culture. Une dose-réponse permettrait d'évaluer la réponse maximale pouvant être obtenue en présence de BMP-9 et de ses peptides dérivés dans les conditions expérimentales de ce projet ainsi que la concentration efficace médiane (EC_{50}). De plus, les doses-réponse du pBMP-9 et du SpBMP-9 n'ont jamais été effectuées, ce qui permettrait d'évaluer les doses de peptides devant être utilisées afin d'obtenir une réponse équivalente à la BMP-9. En effet, les résultats décrits précédemment montrent que les peptides

sembleraient être légèrement moins efficaces que la protéine entière à une concentration équimolaire.

4.2.3 Différenciation à long terme

Lors de cette étude, seul le marqueur ostéogénique précoce Runx2 a été étudié. Par contre, l'expression de Runx2 n'est pas suffisante pour garantir une différenciation en ostéoblastes matures [282]. Une étude de l'expression de gènes ostéogéniques tardifs tels que l'OC et l'OPN et l'évaluation de la minéralisation de l'ECM permettraient de valider la différenciation terminale des MSCs en ostéoblastes matures.

4.2.4 Activation de la voie des Wnt

Outre la voie des Smad1/5/8 et des MAPK étudiées lors de ce projet, d'autres voies de signalisation ont démontré avoir un impact sur la différenciation des MSCs en ostéoblastes, notamment la voie des Wnt. En effet, Tang *et al.* ont démontré que chez des MSCs C3H10T1/2 transfectées par AdBMP-9, la voie des Wnt était activée et permettait une augmentation de l'activité de l'ALP [293]. De plus, l'extinction du gène codant pour la β-caténine inhibe l'activité de l'ALP induite par AdBMP-9 [293]. Par ailleurs, puisque les MSCs ont la capacité de se différencier en plusieurs types cellulaires, il s'avère important d'orienter la différenciation des MSCs vers le phénotype ostéoblastique. En effet, Kang *et al.* ont démontré que les MSCs C3H10T1/2 transfectées par AdBMP-9 pouvaient s'orienter à la fois vers la lignée ostéoblastique et adipocytaire, de façon mutuellement exclusive [133]. Cependant, les résultats de notre étude ont montré que le marqueur adipocytaire PPARγ n'était pas exprimé après 3 jours ce qui indique que les C3H10T1/2 stimulées par la BMP-9 ou ses peptides dérivés ne devraient donc pas être orientées vers le phénotype adipocytaire. De plus, Ross *et al.* ont démontré que l'expression de Wnt-1 inhibait la différenciation en adipocytes des préadipocytes 3T3-L1 [243]. Ces résultats viennent démontrer l'implication de la voie des Wnt dans la différenciation des MSCs en ostéoblastes et la réaction croisée entre la BMP-9 et la voie des Wnt.

4.2.5 Co-immobilisation de peptides

Lors de cette étude, le BMP-9 et ses peptides dérivés ont été utilisés en solution après une adhésion de 4h des cellules. Dans le cas de greffe osseuse, la quantité de BMPs injectées

est élevée afin d'assurer qu'il y ait une quantité suffisante de facteurs de croissance au niveau du site de réparation. Les doses massives utilisées peuvent par contre entraîner de nombreuses complications, notamment le développement d'ossification hétérotopique [67]. Yamachika *et al.* ont comparé la réponse de cellules souches ST2, de préostéoblastes MC3T3-E1 et de myoblastes C2C12 suite à une stimulation de BMP-2 immobilisée ou non sur de l'atélocollagène de type I [321]. Ils ont démontré que l'activité de l'ALP était supérieure en présence de la BMP-2 immobilisée comparativement à la BMP-2 non-immobilisée pour les 3 types cellulaires. Les cellules souches ST2 et les myoblastes C2C12 stimulés par la BMP-2 immobilisée ont montré une expression d'ARNm de marqueurs ostéogéniques (collagène de type I, OPN, OC et l'ALP) supérieure et prolongée et une phosphorylation des Smad1/5/8 prolongée comparativement à la BMP-2 non-immobilisée. Chez les préostéoblastes MC3T3-E1, seule l'expression de l'ARNm de l'ALP était supérieure et prolongée pour les cellules stimulées avec la BMP-2 immobilisée comparé à la BMP-2 non-immobilisée. Par contre, la BMP-2 immobilisée a permis de prolonger la phosphorylation des Smad1/5/8 chez les préostéoblastes MC3T3-E1. L'immobilisation de facteurs de croissance permet aussi d'assurer la stabilité de ceux-ci [181]. La co-immobilisation de peptides d'adhésion et de facteurs de croissance ou de peptides dérivés de ceux-ci pourrait donc être envisagée. He *et al.* ont en effet démontré qu'une co-immobilisation de GRGD et d'un peptide dérivé du BMP-2 (KIPKASSVPTELSAISTLYL) sur un hydrogel de poly(lactide-co-ethylene oxide fumarate) permettait d'augmenter l'activité de l'ALP après 14 jours comparativement à l'hydrogel fonctionnalisé avec seulement RGD ou BMP-2 chez des cellules stromales de moelle osseuse [98]. La co-immobilisation des deux peptides a aussi permis d'obtenir un meilleur niveau de minéralisation et de calcium après 21 jours [98]. De plus, des essais préliminaires effectués par notre groupe ont montré qu'une immobilisation de SpBMP-9 sur des films de PCL permettait un meilleur étalement des MSCs C3H10T1/2 et une expression supérieure de l'ARNm codant pour Dlx5 et Runx2 par rapport à une co-immobilisation de pFibro et de SpBMP-9 (50% mole/mole). Par contre, la co-immobilisation de pFibro et de SpBMP-9 permettait l'adhésion d'un plus grand nombre de cellules par rapport à l'immobilisation de SpBMP-9 seul. La co-immobilisation pourrait donc favoriser la réparation osseuse en plus d'en diminuer les coûts en réduisant les doses utilisées.

4.2.6 Matériaux 3D

Beaucoup d'études sur les biomatériaux utilisent des systèmes 2D. Bien que les systèmes 2D soient utilisés dans le cadre de recouvrement de surface pour des prothèses, ceux-ci ne représentent pas l'environnement dans lequel se trouvent les cellules *in vivo* dans le cas de greffes osseuses [230]. L'utilisation de matériaux 3D serait donc plus représentative, notamment en adoptant une structure poreuse [135,148]. Cependant, les matériaux 3D comportent de nombreux défis, notamment au niveau du contrôle de la porosité, de la colonisation complète du matériau et du transport de nutriments et d'oxygène. De plus, le comportement des cellules diffère sur un matériau en 3D par rapport à un matériau 2D, notamment au niveau de l'interaction des intégrines avec le biomatériau [180]. Par exemple, Grayson *et al.* ont comparé le comportement de hMSCs cultivées sur une lamelle en 2D et une structure 3D de polytéréphthalate d'éthylène [92]. Les cellules cultivées en 3D ont produit une plus grande quantité de protéines de la matrice extracellulaire (collagène de type I et IV, laminine et fibronectine) par rapport aux cellules cultivées sur la surface 2D. Ces protéines étaient organisées sous forme de fibres dans la structure 3D alors que sur la surface 2D, celles-ci étaient peu organisées et se localisaient au niveau du cytoplasme des cellules. Par ailleurs, les cellules cultivées dans la structure 3D exprimaient un niveau supérieur d'intégrines $\alpha_2\beta_1$ par rapport aux cellules cultivées en 2D. De plus, 40% des cellules cultivées en 2D exprimaient les intégrines $\alpha_V\beta_3$ alors que celles-ci étaient absentes chez les cellules cultivées en 3D [92].

CHAPITRE 5. ANNEXE A : SUPPLEMENTARY DATA 1
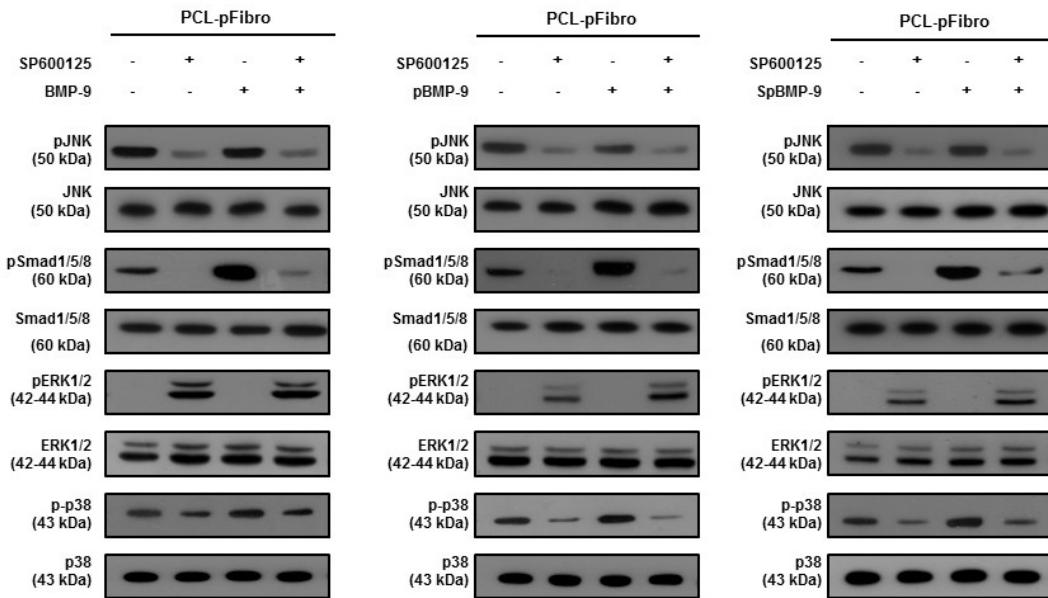

Figure 5.1 The effect of JNK inhibitor SP6001258 on pJNK, ERK1/2, p38 and Smad1/5/8 was studied using western blotting. The C3H10T1/2 cells were seeded on PCL-pFibro for 4h in FBS-free medium and then incubated for 1h with or without 20 μM SP600125 and then stimulated with 1 nM BMP-9, pBMP-9 or SpBMP-9 for 2h in the presence of 1% (v/v) FBS. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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