

Rôle et régulation des intégrines et des cadhérines dans la transdifférenciation MUSCLE/OS en réponse à la BMP-2: approche biomimétique

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THÈSE

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Présentée par

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Rôle et régulation des intégrines et des cadhérines dans la transdifférenciation MUSCLE/OS en réponse à la BMP-2 : approche biomimétique

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Liste des Abréviations

Cellule et environnement cellulaire

BMP, Bone Morphogenetic Protein

BMPR, BMP-Receptors

BRE, BMP Responsive Element

TGF, Transforming Growth Factor

MEC, ECM, Matrice Extracellulaire, Extracellular Matrix

ITG, Integrin

CAD, Cadherin

(h)MSC, (human) Mesenchymal Stem Cell

OSX, Osterix

ALP, Alkaline Phosphatase

Col I, Collagen-I

FN, Fibronectin

OCN, Osteocalcin

ONC, Osteonectin

BSP, Bone Sialo Protein

OPG, Osteoprotegerin

DM, Differentiation Medium

GM, Growth Medium

FBS, Fetal Bovine Serum

Biomatériaux, **Polymères**

PEM, Polyelectrolyte Multilayer (films)

PLL, Poly(L-lysine)

HA, Hyaluronan

EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

s-NHS, N-hydroxysulfosuccinimide

LbL, Layer-by-Layer

GAG, Glycosaminoglycan

Techniques

RT-qPCR, Reverse Transcriptase quantitative Polymerase Chain Reaction

IF, Immunofluorescence

WB, Western Blot

AFM, Atomic Force Microscopy

SEM, Scanning Electron Microscopy

SiRNA, Small Interfering RNA

Table des Matières

Chapitre I. Introduction	
1. REPARATION TISSULAIRE PAR L'UTILISATION DE BIOMATERI	[AUX15
1.1. Développement de trois catégories d'implants	
1.2. Les films à base de biopolymères comme recouvrement de surface ostéoinducteur	
1.2.a. Construction et caractéristiques techniques des films PLL/HA	
1.2.b. Bioactivité des films PLL/HA par chargement de BMP-2	
2 CENERALITES SUR LA RECENERATION TISSULAIRE	20
21 Le tissu une structure complexe et vivante	20
2.1. Le ussu, une structure complexe et vivante	
2.1.a. La matrice extracentulare : centradudage et source d'informations pour les centres	
i Les intégrines récenteurs d'adhésion cellule/matrice	sus
i. Les macginies, récepteurs d'adhésion cellule/cellule	
iii I a transition énithélio-mésenchymateuse : un exemple d'adaptation du répertoire	d'intégrines et
de cadhérines	a integrines et
2.2 Jeux croisés entre les récenteurs adhésifs intégrines et cadhérines	
2.2. seux cloises entre les recepteurs durisits integrations et édulermes	
2.2.h. Formation des systèmes danesnis et interdetions via le cytosquerette	31
3 I F TISSU MUSCUI AIRF · formation différenciation at système adhési	if 3/
3. LE TISSO MOSCOLATINE : IOI mation, unici enclation et systeme auresi 3.1. Equations et correctéristiques du musele	1
3.2. Les facteurs de transcription impliqués dans la muogenèse	
3.2. Les facteurs de transcription impliques dans la différenciation mucculaire	
3.3. Les cadhérines et la différenciation musculaire	
3.3 h Les intégrines et la différenciation musculaire	
4 JETISSU OSSELLY · formation différenciation at système adhésif	
4. LE TISSU OSSEUA : Iorination, uniferenciation et systeme aunesi	40
4.1. Fonctions et caracteristiques de l'os	40
4.2. Les facteurs de transcription impliques dans l'osteoblastogenese	
4.5. Kole et adaptation du systeme adnesit dans la différenciation osseuse	
4.5.a. Les caulerilles et la différenciation osseuse	
4.5.0. Les intégrines et la différenciation osseuse.	
5 COMMUNICATION INTED ODCANES ENTRE I E MUSCLE ET L	
5. COMMUNICATION INTER-ORGANES ENTRE LE MUSCLE ET L'U	JS4/
5.1. Cooperation mecanique et biochimique entre tissu musculaires et tissu osseux	
5.1.a. Une interaction mecanique entre le muscle et l'os	
5.1.b. Les jeux croises biochimiques entre le muscle et l'os	
5.2. Le muscle comme source de cellules souches pour la regeneration osseuse	
5.2.a. Contribution des cellules souches musculaires pour la regeneration osseuse	
5.2.0. Effets de la BIVIP-2 sur les centules musculaires	
i. La madàla C2C12	
A TUNING OF LUI AD DESDONSES TO DMD 2 WITH MATEDIAL SU	
0. IUNING CELLULAR RESPONSES IU DMF-2 WITH MATERIAL SU	KFACES -
revue	
6.1. Introduction	
6.2. Cell responses to soluble BMP-2	
6.2.a. Modulation of BMP-2 signaling at the cell surface	
1. BMP receptor complex formation	
11. Receptor-ligand internalization	
6.2.6. BMP-2 signaling in a cell adhesion context.	
1. Usicogenic and adnesion signaling crosstalk	
11. Effects of BMP-2 on cytoskeleton assembly and cell migration	
0.3. Wimicking the BMP-2 microenvironment with material surfaces	

6.3.a. Temporal control of BMP-2 activity with material surfaces	64
i. Physical entrapment of BMP-2	65
ii. Chemical binding of BMP-2	67
6.3.b. Surface patterning for the spatial control of BMP-2 presentation	69
i. Sub-millimeter patterning of BMP-2	69
ii. Micrometer-sized patterns of BMP-2 on surfaces	70
iii. Nanoscale surface patterning of BMP-2	71
6.3.c. Materials inspired by the interaction of BMP-2 with ECM components	72
i. Modulation of the activity of BMP-2 bound to glycosaminoglycans	73
ii. Co-presentation of BMP-2 and cell binding motifs	75
6.4. Concluding remarks and perspectives	77

Objectifs de la thèse et stratégies......81

1. Résumé	85
1.1. Introduction	85
1.1. Résultats	86
2. Article	87
2.1. Introduction	
2.2. Results	90
2.2.a. Matrix-bound BMP-2/BMPR interaction alters the stiffness response of C2C12 cells	90
2.2.b. β3 integrin is required for cell spreading in response to matrix-bound BMP-2	92
2.2.c. Matrix-bound BMP-2 increases cell migration by affecting cell adhesion site dynamics	96
2.2.d. αvβ3 integrin is required to mediate BMP-2 induced Smad signaling pathway through a S	rc/FAK/
ILK/cdc42 axis	98
2.2.e. β3 integrin regulates Smad stability by repressing GSK3 activity	102
2.3. Discussion	104
2.4. Materials and methods	108
2.6. Supplementary material	116

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2125

1. Résumé	
1.1. Introduction	
1.2. Résultats	
2. Article	
2.1. Introduction	
2.2. Results	130
2.2.a. Matrix bound BMP-2 and soluble BMP-2 drive the muscle-bone transdifferentiatio 2.2.a. bBMP-2 and sBMP-2 induce the expression, secretion and remodeling of bone-spe 2.2.b. bBMP-2 and sBMP-2 induce the expression switch towards Collagen-specific inte 11	n130 cific ECM131 grins and cad- 133
2.2.c. BMP-2 orchestrates integrin-cadherin cross-talk to drive bone differentiation 2.3. Discussion	
2.4. Materiel and methods	144
Conclusions et Perspectives	
Bibliographie	157

Chapitre I. Introduction

1. REPARATION TISSULAIRE PAR L'UTILISATION DE BIOMATERIAUX

1.1. Développement de trois catégories d'implants

Lorsque le corps est lésé, à la suite d'un traumatisme ou d'une pathologie, des mécanismes de réparation s'enclenchent. C'est le cas lors d'une coupure, d'une brûlure, ou encore d'une fracture osseuse. Dans la majorité des cas, le corps peut se réparer de lui-même. Cependant, dans certains cas plus critiques, le défaut nécessite la contribution d'un implant.

Il y a plusieurs catégories d'implants. Les implants les plus faciles à contrôler sont les implants inertes, qui ont uniquement une fonction mécanique. C'est le cas des valves cardiaques, des stents, des implants dentaires ou, dans le domaine orthopédique, des prothèses de hanche ou des prothèses discales lombaires. Ces derniers implants servent uniquement à restaurer une déficience dans l'architecture du squelette (Figure 1) en suppléant une fonction mécanique du corps humain. Ils sont largement utilisés en clinique, apportant des bénéfices considérables pour des risques moindres. Ils sont généralement faits en céramique ou en métal, comme le titane (Geetha et al., 2009). Néanmoins les implants peuvent être améliorés. De nombreuses études visent à améliorer leur intégration dans le corps, en modifiant, par exemple, leur porosité ou leur nature chimique. Une porosité adaptée permet de favoriser la pénétration des cellules du patient au sein de l'implant. Les matériaux mimant la nature biologique du tissu d'origine, comme de l'hydroxyapatite pour mimer l'os, permettent d'améliorer la différenciation des cellules au contact de l'implant. Tous les matériaux ne sont pas utilisables ; les céramiques, par exemple, sont trop fragiles pour être utilisées comme prothèse de hanche.

Dans les cas plus difficiles, par exemple suite à un gros traumatisme lié à un accident ou à un cancer, ou sur un terrain peu favorable, ces implants inertes ne suffisent pas. L'os ne se consolide pas autour de l'implant. Deux stratégies sont alors développées pour activer l'implant et aider le corps à l'intégrer : utiliser des cellules souches (ingénierie tissulaire *ex-situ*) ou utiliser des molécules bioactives (ingénierie tissulaire *in situ*).

Dans la première stratégie, l'ingénierie tissulaire *ex-situ* (Figure 1), des cellules souches sont prélevées chez le patient, cultivées en laboratoire et insérées au sein du biomatériau avant son implantation. Les cellules pré-ensemencées vont à la fois permettre la colonisation de l'implant et la reconstruction du tissu, mais aussi la sécrétion de molécules bioactives pour guider les autres cellules au voisinage du défaut. Cette stratégie prometteuse est soumise à des réglementations très strictes afin d'éviter toute contamination ou dérive des cellules lors du passage en laboratoire. De plus, cela ajoute des contraintes matérielles, à la fois par la nécessité d'intervenir plusieurs fois sur le patient, et par la culture des cellules en laboratoire.

Dans la seconde stratégie, l'ingénierie tissulaire *in situ* (Figure 1), l'implant est fonctionnalisé par des molécules bioactives qui, une fois dans le corps, induisent un recrutement des cellules du patient *via* des récepteurs spécifiques. Ces molécules bioactives vont alors enclencher toute une cascade de réactions au sein des cellules pour les guider dans la réparation de la lésion.



Figure 1 : Les différentes stratégies utilisées pour la régénération osseuse. De haut en bas, un scaffold nu, dont les propriétés mécaniques et la composition chimique peuvent être variés, un scaffold dans lequel des cellules souches sont pré-ensemencées avant l'implantation, ou un scaffold bioactivé par des molécules bioactives telles que des facteurs de croissance.

Dans cette optique, l'équipe du Pr. Catherine Picart développe, depuis 2007, l'utilisation de films multicouches de polyélectrolytes (ou polyelectrolyte multilayers, PEM) comme réservoir de facteurs de croissance, notamment de protéines ostéoinductrices.

1.2. Les films à base de biopolymères comme recouvrement de surface ostéoinducteur

1.2.a. Construction et caractéristiques techniques des films PLL/HA

Les films PEM peuvent être utilisés pour recouvrir les implants afin de les fonctionnaliser. Plusieurs applications de recouvrement par film PEM ont été étudiés, par exemple pour améliorer l'adhésion des cellules sur des vaisseaux artificiels ou des implants de trachée, pour diminuer la coagulation sanguine, pour augmenter la résistance à la dégradation enzymatique d'implants dentaires ou pour diminuer la prolifération des bactéries (Boudou et al., 2010). Leur construction, couche par couche, est relativement simple à mettre en place et permet de recouvrir tout type de matériaux (Decher, 1997) (Figure 2). Elle est basée sur plusieurs types d'interactions, les plus courantes étant les interactions électrostatiques mais également les interactions hydrophobes ou encore les liaisons hydrogènes (Borges and Mano, 2014). Au sein de l'équipe, les biopolymères choisis sont le hyaluronane (HA) et la poly(L-lysine) (PLL). Le HA est un biopolymère chargé négativement et présent dans de nombreux tissus *in vivo*, notamment la peau, le cartilage mais aussi l'os, tandis que la PLL est un polypeptide de l'acide amine lysine, l'un des principaux acides aminés cationiques présents dans notre corps.

Les films natifs, c'est-à-dire tels qu'ils sont à la fin de la méthode d'élaboration, peuvent être stabilisés par la formation de liaisons covalentes entre les bicouches, en utilisant l'agent de réticulation 1-ethyl-3-(-dimethylaminopropyl) carbodiimide (EDC) (Choi et al., 2012) (Figure 2). L'épaisseur du film varie en fonction du nombre de bicouches de PLL/HA (l'épaisseur d'un film de 12 bicouches est d'environ 1 μ m). Par une variation de la concentration d'EDC, le degré de réticulation des films peut être modifié, ce qui va permettre d'obtenir des rigidités différentes (Boudou et al., 2011). Un film faiblement réticulé, c'est-à-dire réticulé par de l'EDC à 30 mg/mL (EDC30), a une rigidité de 200 kPa tandis qu'un film réticulé à 70 mg/mL (EDC70) a une rigidité de 400 kPa (Francius et al., 2006).

1.2.b. Bioactivité des films PLL/HA par chargement de BMP-2

Ces films peuvent être utilisés tels quels ou comme réservoir de molécules bioactives comme des facteurs de croissance (Crouzier et al., 2009). Les facteurs de croissance sont des protéines qui régulent le comportement cellulaire. La famille des Bone Morphogenetic Proteins (BMPs), qui contient 15 BMPs, est une famille de facteurs de croissance initialement découverts pour leur effet sur la formation osseuse (Urist and Strates, 1971). Depuis la découverte de la première BMP en 1965, il a été montré qu'elles étaient aussi impliquées dans de nombreux processus, du développement embryonnaire à la régénération des tissus chez l'adulte (Wagner et al., 2010). La BMP-2, l'une de ces protéines, est connue pour son fort potentiel ostéoinducteur (Yamaguchi et al., 1991). En effet, grâce à des récepteurs spécifiques situés à leur membrane, les cellules souches reconnaissent la BMP-2 et se différencient en cellules osseuses. Les voies de signalisation de la BMP-2 sont détaillées dans la partie 6.

Cette protéine est actuellement utilisée en clinique pour favoriser la régénération osseuse (Schmidmaier et al., 2008). Elle est commercialisée par Inductos® sous la forme d'un kit comprenant une éponge de collagène et 12 mg de BMP-2 à reconstituer. Une fois chargée en BMP-2, l'éponge de collagène est placée au niveau du site du défaut osseux pour aider la régénération lors de fracture de tibia chez l'adulte ou en chirurgie du rachis (AMM : EU/1/02/226/001).

Les films PLL/HA présentent différents avantages : dans les films PLL/HA, la BMP-2 est chargée par simple diffusion (Figure 2) et la quantité incorporée peut être choisie en variant l'épaisseur du film (c'est-à-dire le nombre de bicouches), le taux de réticulation, et la concentration initiale de BMP-2 de la solution de chargement. De plus, les films peuvent être séchés pour augmenter leur stabilité jusqu'à utilisation. Il a été montré que la structure de films chargés en BMP-2 est stable dans le temps et qu'ils sont toujours bioactifs après 1 an (Guillot et al., 2013) ce qui facilite grandement leur utilisation clinique.



Figure 2 : Représentation schématique de la construction des films PEM (PLL/HA). Les polyélectrolytes PLL et HA sont déposés couche par couche (A) afin de former le film natif (B). Ce film est ensuite réticulé par liaisons covalentes (C), le degré de réticulation modulant sa rigidité. La BMP-2 est diffusée à l'intérieur du film réticulé (D).

Une fois bioactivés par la BMP-2, ces films peuvent être utilisés comme recouvrement bioactif de prothèse et ainsi guider les propres cellules du patient vers la reconstruction osseuse autour de l'implant. Leur bioactivité a en effet été validée *in vitro* et *in vivo* (Crouzier et al., 2011a).

Bien que le facteur de croissance BMP-2 soit prometteur pour améliorer la régénération osseuse, il est crucial de comprendre, à l'échelle cellulaire et tissulaire, son rôle dans la différenciation osseuse non seulement pour utiliser cette molécule de manière complétement contrôlée et sans danger pour le patient mais aussi pour éventuellement optimiser le biomatériau.

2. GENERALITES SUR LA REGENERATION TISSULAIRE

2.1. Le tissu, une structure complexe et vivante

2.1.a. La matrice extracellulaire : échafaudage et source d'informations pour les cellules

Les tissus sont des ensembles coopératifs de cellules différenciées qui se caractérisent par une identité territoriale, fonctionnelle et biologique. Les tissus sont constitués non seulement de cellules mais aussi de matrice extracellulaire (MEC). (Figure 3). La MEC est composée essentiellement d'eau, de différents glycosaminoglycanes et de différentes protéines (Alberts et al., 2002). Les plus importantes sont le collagène, la fibronectine, la vitronectine et la laminine. Ces protéines sont communes à de nombreux tissus, mais leur proportion et leur organisation exactes sont propres à chaque tissu et sont liées à leur fonction. Par exemple, le tissu musculaire est composé principalement de laminine tandis que le tissu osseux est composé principalement de collagène I et de fibronectine, ce qui confère au squelette sa rigidité.

La matrice extracellulaire contient aussi des molécules bioactives telles que des facteurs de croissance. Ces molécules peuvent être soit en solution soit liées à la matrice, les protéines de la matrice ayant des domaines d'affinités pour certains facteurs de croissance (Hynes, 2009a).

Lors de la formation d'un tissu, cette MEC est produite, secrétée et arrangée par les cellules elles-mêmes. Puis, réciproquement, la MEC fournit aux cellules différents signaux tels que des signaux biologiques (protéines d'adhésion, facteurs de croissances...) et des signaux mécaniques (rigidité de la matrice, topographie, porosité de l'échafaudage...).

Une cellule est constituée d'une membrane plasmique entourant le cytoplasme. Les cellules eucaryotes possèdent divers éléments dont un noyau et un cytosquelette. Le cytosquelette est une structure dynamique composée de microfilaments, de filaments intermédiaires et de microtubules. Cette structure confère à la cellule non seulement ses propriétés mécaniques et architecturales, mais joue aussi un rôle dans sa différenciation ou sa prolifération. Le noyau renferme le matériel génétique et contrôle tous les éléments de la cellule.

La cellule reconnait les signaux biologiques et physiques de la MEC grâce à différents récepteurs à sa membrane qui transmettent ensuite les informations au noyau. Grâce à des

récepteurs spécifiques, la cellule reconnait la BMP-2 et active la voie de signalisation BMP-2 pour indiquer au noyau que la cellule doit se différencier en cellule osseuse. Grâce à d'autres récepteurs, la cellule adaptera de manière dynamique son cytosquelette selon la rigidité de l'environnement. Cette sensibilité à la rigidité peut affecter la tension du cytosquelette et entraîner une pression mécanique sur le noyau, sa déformation ce qui régule en retour l'expression et/ou l'inhibition de certains gènes (Zajac and Discher, 2008). C'est sous le terme de mécanotransduction que l'on regroupe les voies de signalisation permettant de transformer un signal mécanique en information biochimique. Les récepteurs induisant la mécanotransduction sont des récepteurs d'adhésion.



Figure 3 : Les signaux fournis par le microenvironnement cellulaire. La cellule possède différents récepteurs à sa membrane qui lui permettent de s'ancrer dans son environnement (intégrines et cadhérines) et d'être sensible aux signaux de la MEC (intégrines, récepteurs de molécules de signalisation), tels que les signaux mécaniques (rigidité, porosité, topographie de la matrice) et biochimiques (protéines de la matrice, molécules bioactives...), ainsi qu'aux signaux fournis par les cellules voisines. (Rice et al., 2013)

2.1.b. Les récepteurs d'adhésion à la Matrice Extracellulaire sont caractéristiques des tissus

Les adhésions cellulaires sont fondamentales dans le développement et l'organisation des tissus. Elles permettent soit aux cellules de s'ancrer dans la matrice (adhésion de type cellule/matrice, les intégrines) soit de se lier entre elles (adhésion de type cellule/cellule, les cadhérines).

i. Les intégrines, récepteurs d'adhésion cellule/matrice

Les intégrines sont des protéines transmembranaires découvertes il y a 30 ans par Hynes (Hynes, 1987) et qui permettent à la cellule de s'intégrer dans son microenvironnement. Elles sont composées d'un long domaine extracellulaire qui lui permet de s'attacher aux protéines de la matrice, et d'un court domaine cytoplasmique qui est relié au cytosquelette.

L'intégrine est une unité hétérodimérique composée d'une sous unité α (ITGA) et d'une sous unité β (ITGB) liées de façon non covalente. A ce jour, 18 chaines α et 8 chaines β sont répertoriées, pouvant former 24 combinaisons différentes. Chaque combinaison reconnaît spécifiquement une ou plusieurs protéine(s) de la matrice et permet à la cellule de s'y attacher (Figure 4). Par exemple, l'intégrine $\alpha 2\beta 1$ se lie au collagène, tandis que l'intégrine $\alpha 7\beta 1$ se lie à la laminine.

Comme chaque tissu possède sa propre composition de protéines matricielles, les cellules de chaque tissu possèdent aussi leur propre répertoire d'intégrines.



Figure 4 : Représentation schématique des intégrines et de leur ligand. Les chaînes β , souvent plus communes et moins spécifiques que les chaînes α , sont représentées au centre du système. Chaque combinaison d'une chaîne α et d'une chaîne β forme un récepteur pour une protéine de la matrice telle que le collagène, la laminine, ou une protéine contenant un motif RGD comme la fibronectine. (Zent and Pozzi, 2010)

La signalisation passant par les intégrines est une signalisation bidirectionnelle. La fonction d'une intégrine dépend de son état d'activation (état déplié) ou inactivation (état replié) correspondant à la capacité de l'intégrine à changer son état conformationnel et à interagir avec la MEC selon le partenaire cytoplasmique (signalisation inside-out) (Figure 5). Ce dépliement augmente l'affinité du domaine extracellulaire de l'intégrine à son ligand. Dès lors, les intégrines se rassemblent pour former un cluster à la membrane plasmique, générer la formation de sites d'ancrage à la matrice et transmettre des signaux extracellulaires à l'intérieur de la cellule, en passant par le cytosquelette (signalisation outside-in).



Figure 5 : Activation des intégrines, signalisation inside-out outside-in. La signalisation inside-out provient de la cellule elle-même et indique à l'intégrine qu'elle doit passer d'un état replié à un état déplié, et ainsi se lier aux protéines de la matrice. L'adhésion des intégrines au ligand induit leur regroupement en cluster, permettant ainsi de transmettre les signaux de l'extérieurs vers la cellule, c'est-à-dire la signalisation outside-in. (Askari et al., 2009)

Les intégrines sont les protéines d'adhésions qui permettent à la cellule d'être sensible à la rigidité de la matrice. Ce processus est principalement dû à une variation de la tension du cytosquelette en fonction de la tension extérieure. Dans une MEC suffisamment rigide, les intégrines se regroupent et transmettent des forces de tension plus grandes au cytosquelette, par la formation de fibres de stress (Paszek et al., 2005; Riveline et al., 2001) Les voies de signalisation associées à ROCK, MRCK ou mDia ont été décrites pour réguler la contractilité cellulaire via la réorganisation du cytosquelette d'actine, élément essentiel dans la réponse et l'adaptation de la cellule aux propriétés physiques de son environnement (Burridge and Wittchen, 2013; Jégou et al., 2013; Ronan et al., 2015; Totsukawa et al., 2004). Cependant, les mécanismes impliqués dans la reconnaissance de la rigidité par les intégrines ne sont pas encore bien élucidés. Elosegui-Artola et al., montrent que les intégrines transmettent

l'information mécanique par leur constante d'association/dissociation à la MEC (Elosegui-Artola et al., 2014). Ainsi, selon la rigidité de la MEC, la cellule adapte son répertoire d'intégrines pouvant expliquer en partie pourquoi la cellule possède différents récepteurs d'intégrines pour une même protéine de la MEC.

ii. Les cadhérines, récepteurs d'adhésion cellule/cellule

Les cadhérines, glycoprotéines transmembranaires calcium-dépendantes, sont une des familles de récepteurs responsables des jonctions cellule/cellule. L'existence de protéines calcium-dépendantes impliquées dans les adhésions cellule/cellule a été mise en évidence en 1977 (Takeichi, 1977) mais la première cadhérine à avoir été isolée et identifiée fut la E-cadhérine, en 1981. Depuis, plus de 350 cadhérines ont été découvertes, en incluant les différentes isoformes (Hulpiau and van Roy, 2009). Tout comme les intégrines, les cadhérines possèdent un domaine extracellulaire et un domaine cytoplasmique. Celui-ci est relié au cytosquelette *via* les α , β et γ catenines (Cheng et al., 1998). Cependant, contrairement à une intégrine, les cadhérines forment des complexes homodimèriques. Leur domaine extracellulaire peut se lier au domaine extracellulaire d'une cadhérine de même nature d'une cellule voisine (Figure 6).



Figure 6 : Structure d'une cadhérine et lien avec le cytosquelette. La cadhérine/cadhérine est une unité homodimère. La partie cytoplasmique de la cadhérine est reliée au cytosquelette *via* les α , β et γ catenines. Adapté de (Yonemura et al., 2010)

Historiquement, les cadhérines étaient répertoriées en fonction du tissu dans lequel elles ont été identifiées, leur nom se rapportant ainsi à la lettre de ce tissu (Table 1). Par exemple, la M-cad a été identifiée dans les cellules musculaires, d'où le M. Cependant, lorsque 8 cadhérines ont été découvertes en même temps, grâce à des techniques de RT-PCR (Suzuki et al., 1991), il a été proposé de leur donner un numéro. Bien que chaque tissu possède son

Cadherin	Specie(s)	Tissue(s)	Reference(s)
E-cad – CDH1	Xenopus	Early Embryo and epithelia	(Hyafil et al., 1981)
N-cad – CDH2	Chicken, human, <i>Xenopus</i>	Neuronal and mesodermal cells	(Miyatani et al., 1989; Nose and Takeichi, 1986)
P- cad – <i>CDH3</i>	Mouse, human	Placenta	(Miyatani et al., 1989; Nose and Takeichi, 1986)
R-cad – CDH4	Chicken	Retina	(Suzuki et al., 1991)
VE-cad – CDH5	Bovine	Endothelial cells (Vessels)	(Suzuki et al., 1991)
OB -cad – <i>CDH11</i>	Xenopus	Brain, Retina, Osteoblasts	(Okazaki et al., 1994; Suzuki et al., 1991)
M -cad – <i>CDH15</i>	Mouse	Muscle	(Liaw et al., 1990)

propre répertoire de cadhérines, les cadhérines peuvent jouer des rôles cruciaux dans d'autres tissus. C'est pourquoi cette seconde nomenclature peut être préférable dans certains cas.

 Table 1 : Exemples de tissus où les cadhérines ont été identifiées. Adapté de (Kemler, 1992)

Récemment, le rôle mécanosenseur des cadhérines a été mis en évidence (Ganz et al., 2006). Les jonctions cellule/cellule sont déstabilisées sur des substrats rigides et favorisées sur des substrats mous (Ladoux et al., 2010; You et al., 2013). De plus, les cadhérines ne permettent pas toutes la même tension sur le cytosquelette. Par exemple, la tension résultant d'une jonction N-cad/N-cad est comparable à celle d'une liaison intégrines/ECM (Chopra et al., 2011). Le répertoire de cadhérines disponible pour la cellule permet donc des tensions plus ou moins fortes sur le cytosquelette.

iii. La transition épithélio-mésenchymateuse : un exemple d'adaptation du répertoire d'intégrines et de cadhérines

Les récepteurs d'adhésion contribuent à faire la spécificité d'un tissu et jouent un rôle crucial dans les phénomènes physiologiques et pathologiques. Par exemple, lors de la différenciation cellulaire, la composition de la MEC change et les cellules adaptent leur répertoire de récepteurs d'adhésion à ce nouvel environnement, aussi bien pour s'adapter à la nature des protéines qu'à la rigidité de la nouvelle matrice. La transition épithélio-mésenchymateuse est un processus impliquant un changement de répertoire de ces récepteurs d'adhésion. Lors de la transition épithélio-mésenchymateuse, les cellules des tissus épithéliaux se désolidarisent du tissu et sont de nouveau capable de migrer et de proliférer. Ce processus très étudié intervient aussi bien dans des conditions physiologiques, comme l'embryogenèse ou la

cicatrisation, que dans des conditions pathologiques, comme l'apparition de métastases. De plus, il s'agit d'un exemple typique de plasticité cellulaire. Des signaux engendrent une baisse de l'expression de certaines cadhérines au bénéfice d'autres, ce qui entraîne une diminution de la cohésion du tissu (Christofori, 2003). Les cellules produisent de nouveau des protéines de la MEC et, en augmentant l'expression de leurs intégrines, elles peuvent recommencer à migrer (Radisky, 2005).

Ces deux types d'adhésion ont longtemps été étudiés séparément. Cependant, étant toutes les deux reliées au cytosquelette, elles partagent les mêmes acteurs au sein de la cellule pour réguler différentes fonctions cellulaires. C'est pourquoi le terme « adhesive crosstalk », que l'on peut traduire par « jeux croisés des systèmes adhésifs », est communément utilisé pour mettre en évidence les possibles communications entre ces deux systèmes distincts (Weber et al., 2011).

2.2. Jeux croisés entre les récepteurs adhésifs intégrines et cadhérines

L'équipe de DeSimone, dans leur revue de 2011, répertorie quelques exemples connus de coopérations entre les intégrines et les cadhérines (

Table 2, extraite de (Weber et al., 2011)).

Certaines portent sur les processus de transition épithélio-mésenchymateuses décrits dans la partie 2.1.b.iii. De plus, ce tableau révèle que les jeux croisés les plus étudiés à ce jour concernent la E-cad et la N-cad. Les interactions intégrine/cadhérine impliquant la cad-11 (ou OB-cad) n'étaient alors pas connues. La première preuve de la coopération entre cad-11 et les intégrines fut apportée par Langhe *et al.* (Langhe et al., 2016). Ils montrent que cad-11 est nécessaire à la phosphorylation et au recrutement de la paxilline aux nouvelles adhésions focales, ce qui n'est pas le cas de N-cad ou de C-cad. De plus, lorsque cad-11 est localisée dans les plaques focales d'adhésion, son domaine cytoplasmique favorise la liaison de la plaque focale au cytosquelette.

Cadherin	Integrin	Cell or tissue type	Cellular or physiological condition	References
		MCF10A mammary epithelial	Proliferation	(Fournier et al., 2008)
	β1	Human colon carcinoma Moser cells	Epithelial- mesenchymal transition	(Wang et al., 2004)
	$\alpha 3/\beta 1$ Immortalized r kidney epitheli	Immortalized mouse kidney epithelia	Cell-cell adhesion	(Chattopadhyay et al., 2003)
	$\alpha 5/\beta 1$ Sal	Salivary gland	Branching morphogenesis	(Onodera, 2010; Sakai et al., 2003)
E-cad	-cad $\alpha V \text{ ou} \\ \alpha 5/\beta 1$	L fibroblasts, S180 mouse sarcoma, SCC13 squamous cell carcinoma	Cell-cell adhesion	(Martinez-Rico et al., 2010)
	αV	Human colonic adenocarcinoma	Cell migration	(Canonici et al., 2008)
	$\alpha 3 \text{ or } \beta 1$	Ovarian carcinoma cells	Epithelial- mesenchymal transition	(Symowicz et al., 2007)
	Fibronectin receptorFisher rat thyroid (FRT) cells $\alpha3$ and $\alpha6$ HT-29 human colon adenocarcinoma	Fisher rat thyroid (FRT) cells	Cell-matrix adhesion	(Balzac et al., 2005)
		Cell-cell adhesion	(Chartier et al., 2006)	
	β1	PC12, chick neural retinal explants	Neurite outgrowth	(Arregui et al., 2000)
N-cad	β1, β3	Neural crest cells	Cell migration	(Monier-Gavelle and Duband, 1997; Theveneau et al., 2010)
DE-cad	β	Drosophila border cells	Collective cell migration	(Llense and Martín-Blanco, 2008; Wang et al., 2010)
VE-cad	$\alpha V/\beta 3$	Bovine aortic endothelial cells	Inflammation	(Liu et al., 2008b)

Table 2 : Exemples d'intera	ctions connus entre intégrines et ca	dhérines. Adapté de (Weber et al., 201	1)
1	8		

2.2.a. Formation des systèmes adhésifs et interactions via le cytosquelette

Au niveau des intégrines, la cellule forme des structures d'adhérence composées de 156 protéines pouvant être classées en trois catégories :

- Les <u>protéines adaptatrices</u> qui permettent de faire la liaison entre les intégrines et le cytosquelette d'actine. On y trouve par exemple la taline, la vinculine ou la paxilline ;
- Les protéines de signalisation, régulant la dynamique des sites d'adhésion. C'est le cas des tyrosines kinases, telles que Src, FAK et PI3K, et des phosphatases. FAK se lie à la taline et à la paxilline et peut activer les intégrines.

 Et les protéines qui jouent un rôle sur l'organisation du cytosquelette d'actine : les <u>RhoGTPases</u> dont les principaux membres sont RhoA (Ras homolog gene family, member A), Rac1 (Ras-related C3 botulin toxin substrate 1) et Cdc42 (Cell division control protein 42 homolog). Les RhoGTPases peuvent être régulées par des protéines de signalisation telles que FAK.

Dans un premier temps, la cellule forme des structures d'adhérence naissantes et des complexes focaux (Zaidel-Bar et al., 2004). Ces petites structures, de moins de 1 μ m, sont très dynamiques avec une durée de vie d'environ 60 s. Sous l'action des RhoGTPases, notamment de RhoA et de ROCK, ces complexes focaux se rassemblent et fusionnent en adhérences focales, caractérisées par le recrutement de la vinculine, de la paxilline et des phosphoprotéines comme FAK. Les adhérences focales sont des structures plus allongées et moins dynamiques que les complexes focaux. Elles ont une taille d'environ 2 μ m sur 3 à 10 μ m et une durée de vie de 30 à 90 min.

Ce système adhésif permet à la cellule de migrer et de proliférer. Lorsqu'elle rencontre une autre cellule, elle commence à former les premiers contacts cellule/cellule, c'est-à-dire les cadhérines. Les GTPases sont alors recrutées aux cadhérines et les adhésions focales sont réorganisées afin de transmettre les forces aux cadhérines (Braga et al., 1997). Ceci est le premier jeu croisé entre les intégrines et les cadhérines.

Les forces de traction du cytosquelette d'actine seraient alors réparties entre les intégrines et les cadhérines (Figure 7).



Figure 7 : Répartition des tensions du cytosquelette entre les cadhérines et les intégrines. La coopération intégrine/cadhérine permet de maintenir une tension du cytosquelette. (Collins and Nelson, 2015)

Partage des protéines adaptatrices :

La vinculine est une protéine adaptatrice des structures d'adhésion. Elle relie la taline, qui est attachée aux intégrines, à l'actine. De par sa localisation, la taline a un rôle crucial dans la réponse des adhésomes aux contraintes mécaniques et elle contrôle l'assemblage et le désassemblage des adhésions focales (Galbraith et al., 2002). Or la vinculine est aussi présente dans les jonctions intercellulaires. Lorsque la tension intracellulaire est forte, la vinculine est recrutée aux cadhérines pour se lier à la protéine α -catenin et ainsi renforcer les jonctions cellule/cellule (Yonemura et al., 2010) (Figure 8).

Partage des protéines de signalisation :

FAK est un autre acteur des adhésomes qui est commun aux jonctions cellule/cellule. Une inhibition de FAK entraîne une perturbation des adhésions N-cad (Schaller, 2004). De plus, la formation des adhésions, aussi bien cellule/cellule que cellule/matrice, entraînerait l'activation des protéines de la famille Src (Weber et al., 2011).



Figure 8 : Recrutement de la vinculine aux cadhérines. Lorsque la cellule est isolée, la myosine n'agit pas sur le filament d'actine et l' α -catenin adopte une conformation repliée. La liaison de la cadhérine à une cellule adjacente entraîne sa traction, ce qui permet à la myosine d'agir en retour sur le filament d'actine et d'étirer l' α -catenin. La vinculine peut alors se lier à l' α -catenin, renforçant ainsi la liaison des filaments d'actine à l' α -catenin. L'adhésion est ainsi augmentée. (Yonemura et al., 2010)

Partage des RhoGTPases :

Concernant les RhoGTPases, Rho est activé par la formation d'intégrine ce qui va permettre la formation des adhésions cellule/cellule. Cependant, une trop forte activation de Rho déstabilise les cadhérines (Zhong et al., 1997). La formation des cadhérines augmente l'activité de Rac1 mais leur maintien dans le temps conduit à son inhibition (Noren et al., 2001).

Enfin, les activités de PI3K et Rac, qui sont respectivement une kinase et une GTPase, sont à la fois stimulées par les intégrines et réprimées localement par la N-cad (Mui et al., 2016).

Les effets des adhésions cellule/cellule et cellule/matrice sur la tension du cytosquelette d'actine ne sont pas forcément antagonistes. Ces systèmes adhésifs peuvent aussi agir en coopération et sont dépendants l'un de l'autre pour assurer une homéostasie tissulaire correcte : les intégrines peuvent favoriser la stabilité des cadhérines et vice-versa.

Les interactions intégrine/cadhérine *via* le cytosquelette entrainent une collaboration des intégrines et des cadhérines dans la réponse de la cellule aux contraintes mécaniques. La voie YAP/TAZ pourrait être impliquée dans ces interactions indirectes entre les intégrines et les cadhérines lors de la mécanotransduction. YAP et TAZ sont des coactivateurs transcriptionnels impliqués dans la réponse à une contrainte mécanique (Dupont et al., 2011)

et sont à la fois régulés par les contacts cellule/cellule et par les contacts cellule/matrice (Dupont, 2015; Piccolo et al., 2014).

2.2.b. Effets des interactions intégrine/cadhérine sur la MEC

Les systèmes adhésifs interagissent donc entre eux au sein de la cellule, *via* de nombreux acteurs qui peuvent jouer sur le cytosquelette d'actine. Nous allons maintenant voir les effets des interactions des systèmes adhésifs sur la MEC.

Ces systèmes adhésifs ont un rôle dans la production des protéines de la matrice. Dans le processus pathologique de la fibrose, un excès de matrice extracellulaire est produit et déposé dans les tissus. Cela conduit à la formation de tissu cicatriciel, à la perturbation de l'architecture normale d'un tissu et à la dégradation de l'organe. Par un rôle indirect sur le facteur de croissance Transforming Growth Factor- β (TGF- β), de la famille des Transforming Growth Factor, qui lui-même favorise la production du collagène dans les phénomènes de fibrose, l'intégrine $\alpha V\beta 6$ a un rôle important sur la fibrose (Agarwal, 2014). Une autre intégrine, l'intégrine $\alpha 3\beta 1$, semble aussi être importante dans ce phénomène puisque son inhibition freine le processus de fibrose.

Il est intéressant de noter que non seulement les intégrines, mais aussi les cadhérines, semblent participer à la fibrose. En particulier, cad-11 semble participer à la fibrose de la peau et des poumons. Son expression est augmentée dans les cellules de lal peau fibrosée et les souris déficientes en cad-11 présentent moins de phénomène de fibrose. Cependant, le mécanisme par lequel cette cadhérine régule la fibrose n'est pas encore connu (Agarwal, 2014).

Une fois secrétées par les cellules, les protéines de la MEC sont assemblées et remodelées en fibres et/ou en réseau. Etant en contact direct avec la MEC, les intégrines sont les récepteurs les plus généralement connus pour participer à son remodelage. En se liant à la fibronectine, l'intégrine $\alpha 5\beta 1$ permet non seulement à la cellule d'adhérer à la matrice, mais participe aussi à la réorganisation de la fibronectine en fibres. L'intégrine $\alpha 5\beta 1$ induit l'étirement de la fibronectine, révélant des sites importants pour l'auto-assemblage de la fibronectine en fibres (Mao and Schwarzbauer, 2005). De plus, par ce processus, d'autres sites sur la fibronectine sont révélés, notamment des sites de liaison à d'autres protéines de la MEC telles que le

collagène. Indirectement, les intégrines participent donc aussi à l'assemblage du collagène en fibres (Figure 9).

Dans la pathologie de l'angiome caverneux, aussi appelé malformation caverneuse cérébrale, une activation de l'intégrine β 1 serait responsable non seulement d'une désorganisation de la fibronectine en provoquant l'alignement des fibres de fibronectine suite à une forte activité de Rho-ROCK mais aussi d'une perte des contacts cellule/cellule via les E-cadhérines (Faurobert et al., 2013).



Figure 9 : Etirement de la fibronectine par les intégrines et auto-assemblage en fibres. L'adhésion de l'intégrine aux fibres de fibronectine induit leur étirement ce qui révèle des sites importants pour leur auto-assemblage. En vert : les fibres d'actine. (Mao and Schwarzbauer, 2005)

Les cadhérines aussi semblent avoir un rôle dans le remodelage de la matrice extracellulaire. Tout d'abord un rôle indirect *via* le cytosquelette : l'intégrine a besoin d'une tension cellulaire suffisante pour pouvoir étirer la fibronectine et les cadhérines participent à maintenir cette tension (Dzamba et al., 2009) (Figure 10). Le transfert des tensions des cadhérines dans le processus de fibrillogenèse (assemblage en fibres) de la fibronectine est dépendant de Rac, qui est une RhoGTPase.



Figure 10 : Rôle des Cadhérines dans la tension nécessaire à la fibrillogenèse. Les cadhérines permettent de maintenir la tension du cytosquelette qui est importante dans les processus de remodelage de la fibronectine. (Dzamba et al., 2009)

Cependant les interactions peuvent aussi être directes. Comme mentionné précédemment, cad-11 a un rôle direct dans le remodelage de la fibronectine. Cette cadhérine, récemment trouvée dans les plaques focales d'adhésion, favorise l'adhésion des intégrines à la fibronectine (Langhe et al., 2016). Cependant, toutes les cadhérines ne favorisent pas le remodelage de la fibronectine. C'est le cas de N-cad qui, en stabilisant la forme inactive d' α 5, inhibe la fibrillogenèse (Julich et al., 2015).

Les jeux croisés entre les intégrines et les cadhérines sont nombreux et participent à la sécrétion et au remodelage de la MEC. La MEC étant propre à chaque tissu, nous allons maintenant nous focaliser sur la formation des tissus musculaires et osseux, et sur les systèmes adhésifs dans ces tissus.

3. LE TISSU MUSCULAIRE : formation, différenciation et système adhésif

3.1. Fonctions et caractéristiques du muscle

Le muscle est un tissu fondamental et l'un des plus abondants chez les vertébrés. Il représente 30 à 40 % de la masse du corps humain. La MEC musculaire peut être séparée en deux parties : la matrice interstitielle et la matrice péricellulaire. La matrice interstitielle, servant à maintenir le tissu, est principalement composée de collagènes, d'élastine, de fibronectine et de tenascine. La matrice péricellulaire, en contact direct avec les cellules, contient la membrane basale qui est composée de laminine, de collagène IV, et qui sert à séparer les cellules musculaires de la matrice interstitielle (Thorsteinsdóttir et al., 2011).

Les syndecans font aussi partie de la MEC musculaire. Ces protéoglycanes peuvent se lier directement aux protéines de la MEC ainsi qu'aux facteurs de croissance (Thorsteinsdóttir et al., 2011). Ils sont donc importants dans les jeux croisés intégrines/récepteurs de facteurs de croissance.

La MEC joue un rôle crucial dans les différentes étapes de la myogenèse (Thorsteinsdóttir et al., 2011). Par exemple, une mutation sur le gène codant pour la laminine-111 entraîne une dystrophie musculaire congénitale caractérisée par une faiblesse musculaire et une hypotonie.

Le muscle est responsable de nombreuses fonctions volontaires telles que la motricité, le maintien de la posture, mais aussi des fonctions involontaires comme la digestion, la respiration ou l'activité cardiaque.

Il existe 3 types de tissus musculaires :

- Le tissu musculaire lisse qui tapisse les organes viscéraux creux et les organes respiratoires ;
- Le tissu musculaire strié cardiaque (le myocarde) ;
- Et le tissu musculaire strié squelettique.

Le tissu musculaire strié squelettique est attaché au squelette osseux et permet tous les mouvements volontaires. Ce muscle est composé de fibres d'un diamètre de 10 à 100 μ m et d'une longueur pouvant aller jusqu'à 30 cm. Ces fibres sont multinuclées, c'est-à-dire qu'elles possèdent plusieurs noyaux. Ceci est dû au fait qu'elles sont formées par la fusion

de plusieurs cellules progénitrices musculaires entre elles. Ces cellules progénitrices, nommées myoblastes, sont retrouvées aussi chez l'adulte afin de réparer le tissu musculaire squelettique en cas de blessure. Lorsque le tissu musculaire a besoin d'être réparé, les cellules satellites migrent au site de lésion, prolifèrent et se différencient pour fusionner en fibres musculaires, appelées myotubes. Le processus de différenciation musculaire, appelée myogenèse, est régulé par différents facteurs de transcription.

3.2. Les facteurs de transcription impliqués dans la myogenèse

Un facteur de transcription est une protéine qui, transloquée au noyau, active et/ou réprime certains gènes afin de réguler le comportement de la cellule, notamment sa différenciation. Les facteurs de transcription nécessaires à la myogenèse font partie de la famille des *myogenic regulatory factors* (MRF). Les membres de cette famille sont myf-5, myoD, myogenin, et MRF4 et ils interviennent tous à des étapes spécifiques (Sabourin and Rudnicki, 2000).

Initialement, la cellule exprime le facteur de transcription Pax7. Quand elle s'engage en voie de différenciation, l'expression de Pax7 diminue et myoD est exprimée transitoirement. Lors de la différenciation, Myogenin est exprimée progressivement et permet la fusion des cellules satellites en myotubes (Figure 11). Tous ces facteurs de transcription sont utiles à des stades spécifiques de la myogenès (Olguin et al., 2007).



Figure 11 : L'intervention des facteurs de transcription myogéniques selon l'étape de différenciation. Pax7 est exprimé jusqu'à l'entrée des cellules satellites en différenciation. MyoD et myf-5 sont ensuite requis pour l'engagement des cellules en différenciation myogénique. Les cellules deviennent alors des myoblastes qui peuvent proliférer et se différencier en myofibres sous l'action de myogenin et de MRF-4. Adapté de (Sabourin and Rudnicki, 2000)
Il a été récemment montré que les cellules musculaires sont capables de se dédifférencier et de revenir à l'état de myoblaste. Sous l'effet du facteur de transcription Msx2, les myotubes se cellularisent et recommencent à se multiplier. De plus, le niveau d'expression de myogenin diminue (Figure 12, (Yilmaz et al., 2015)). Il est donc envisageable que lors d'une lésion musculaire, non seulement les cellules satellites mais aussi les fibres musculaires adultes participent à la régénération du muscle.



Figure 12 : Dédifférenciation des myotubes. Sous l'effet de Msx2, les myotubes se cellularisent, expriment de nouveau des marqueurs myoblastiques, et peuvent recommencer à proliférer. Adapté de (Yilmaz et al., 2015)

3.3. Rôle et adaptation des systèmes adhésifs dans la différenciation musculaire *3.3.a. Les cadhérines et la différenciation musculaire*

Le répertoire de cadhérines change durant la myogenèse, et chacune est importante à une étape spécifique. La cad-11 est exprimée faiblement très précocement, tandis que la N-cad et la M-cad sont exprimées plus longuement (Charrasse et al., 2003; Padilla et al., 1998). N-cad est importante pour l'arrêt du cycle de division cellulaire et l'enclenchement de la différenciation myogénique, c'est pourquoi elle est réprimée en fin de myogenèse, tandis que M-cad, exprimée plus tardivement, intervient dans les dernières étapes de la myogenèses (Figure 13, (Charrasse et al., 2003)).



Figure 13 : Représentation schématique de l'intervention des cadhérines durant les différentes phases de la différenciation musculaire. Cad-11 est trouvée précocement mais son expression est rapidement perdue. En revanche, N et R-cad sont exprimées durant toute la première myogenèse. M-cad est exprimée durant la seconde myogenèse et, avec N-cad, est maintenue chez l'adulte. (Charrasse et al., 2003)

Les cadhérines interviennent durant la myogenèse notamment en régulant les Rho-GTPases. L'adhésion N-cad entraîne une augmentation de l'activité de RhoA et une diminution des activités de Rac1 et Cdc42. Or ces RhoGTPases ont un rôle activateur et inhibiteur de la myogenèse, respectivement (Charrasse et al., 2003).

3.3.b. Les intégrines et la différenciation musculaire

Des délétions d'intégrines chez la souris ont révélé l'importance de trois intégrines dans la formation du muscle :

- La chaîne β1, dont le Knock Out (KO) entraîne une réduction de la masse musculaire et la mort des souris rapidement après la naissance (Mayer, 2003). De plus, des expériences plus poussées montrent que deux formes d'intégrines β1 sont importantes, β1A et β1D, et chacune à différentes étapes de la myogenèse (Baudoin et al., 1998) (Figure 14) ;
- La chaîne α5, dont le KO entraîne une dystrophie des muscles (Taverna et al., 1998);

 et la chaîne α7, dont le KO entraîne une dystrophie musculaire progressive (Mayer et al., 1997).

Concernant les chaînes β , il a longtemps été supposé qu'uniquement β 1 avait un rôle dans la myogenèse. Cependant, Liu *et al.*, ont montré que β 3 est exprimée transitoirement dans les cellules satellites lors de la myogenèse et que son inhibition entraîne une diminution de l'expression de la myogenin à 48 h (Liu et al., 2011a).

Les intégrines $\alpha 4\beta 1$, $\alpha 4\beta 7$, αV , $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$ ont un rôle majeur dans la myogenèse et sont retrouvées dans les myoblastes (Mayer, 2003). Les cinétiques des expressions des intégrines et leur rôle lors de la myogenèse ont été étudiés dans la littérature.

 α 5 β 1 et α 6 β 1 sont réprimées après la formation des myotubes, tandis qu' α 7 β 1 est fortement augmentée durant la fusion des myoblastes (Mayer, 2003). α 5 β 1 et α 6 β 1 coexistent donc à la même période, mais auraient des rôles différents. α 5 maintiendrait la prolifération des myoblastes tandis qu' α 6 induirait leur différenciation. De plus, α 7 β 1 serait importante pour la migration des myotubes. En effet, bien qu' α 3, α 6 et α 7 β 1 soient les trois intégrines spécifiques de la laminine, seule α 7 β 1 permet un attachement suffisant du cytosquelette à la MEC pour la stabilité musculaire lors de la contraction (Mayer, 2003). De plus, des myoblastes avec un niveau augmenté d' α 7 β 1 présentent un meilleur attachement à la laminine et prolifèrent plus rapidement, tout en se différenciant normalement vers le muscle (Liu et al., 2008a).

Enfin, des thérapies commencent à émerger pour soigner la dystrophie musculaire congénitale de Duchenne en utilisant le rôle d' α 7 β 1. Des injections de laminine-111 dans des modèles de souris pour cette dystrophie permettent d'augmenter le niveau de l'intégrine α 7, et ainsi de prévenir les dommages musculaires liés à la dystrophie musculaire congénitale (Riederer et al., 2015; Rooney et al., 2009).



Figure 14 : Représentation schématique de l'expression des différentes intégrines dans les cellules musculaires squelettiques. Dans les myoblastes en migration, les chaînes $\alpha 4$ à $\alpha 7$, αV et $\beta 1A$ sont présentes. Durant la seconde myogenèse, les chaînes α sont les mêmes, mais la chaîne $\beta 1A$ est remplacée par la chaîne $\beta 1D$. Chez l'adulte, uniquement $\alpha 7\beta 1D$ est trouvée dans les membranes des fibres musculaires striées et aux jonctions tendineuses (MTJ), tandis qu'aux jonctions neuronales (NMJ), les chaines $\alpha 3$, $\alpha 7$ et αV sont présentes. (Mayer, 2003)

4. LE TISSU OSSEUX : formation, différenciation et système adhésif4.1. Fonctions et caractéristiques de l'os

Le tissu osseux représente 15 % de la masse du corps humain. L'os est un tissu particulier car il comporte une phase minérale (75 %). Celle-ci est principalement constituée de calcium, sous forme d'hydroxyapatite et de carbonate de calcium, et d'autres minéraux comme le fluor, le potassium et le magnésium.

La MEC de la phase organique de l'os est composée majoritairement de collagène I (90 %). Le collagène est une protéine sécrétée par les ostéoblastes et auto-assemblée en fibres. Ce processus est nécessairement long afin d'obtenir, *in fine*, une structure osseuse bien ordonnée, et donc plus solide. D'autres protéines non-collagéniques composent aussi la MEC osseuse. C'est le cas des protéoglycanes, de l'ostéopontine (OPN) qui relie l'hydroxyapatite aux cellules osseuses, de l'ostéonectine (ONC) et de l'ostéocalcine (OCN) qui interviennent dans la minéralisation, et de la sialoprotéine osseuse (BSP, pour Bone SialoProtein) qui est aussi pro-minéralisante. L'osteocalcine est une hormone ayant un rôle prépondérant dans la communication inter-organes (Karsenty et al., 2016).

La MEC osseuse contient aussi de nombreuses molécules bioactives telles que des cytokines ou des facteurs de croissance, qui sont libérées lors de sa dégradation par les cellules.

Le tissu osseux a quatre principales fonctions :

- Une fonction mécanique. Il supporte le corps et les contraintes mécaniques, et protège les organes ;
- Une fonction métabolique, en stockant les sels minéraux qui sont importants dans de très nombreux processus biologiques ;
- Une fonction hormonale via la sécrétion de l'ostéocalcine (Karsenty and Ferron, 2012);
- Et une fonction hématopoïétique, car il sert de support à la moelle osseuse, contenant les cellules souches hématopoïétiques qui permettent de régénérer le sang.

Les os sont séparés en trois catégories :

- Les os courts, comme les os du carpe ;
- Les os plats, tels que le sternum ou les côtes ;
- Et les os longs comme le tibia ou le fémur.

Les os longs sont composés d'une partie d'os spongieux aux extrémités, d'une partie d'os cortical ou compact, et sont entourés du périoste (Figure 15). A l'intérieur des os longs se trouve de la moelle osseuse, renfermant les cellules souches mésenchymateuses.



Figure 15 : Les différentes parties d'un os long. Les extrémités sont constituées d'os spongieux. Au centre, l'os compact renferme la moelle osseuse et est entouré du périoste.

En effet, en plus des cellules souches hématopoïétiques, l'os héberge aussi les cellules souches mésenchymateuses (notées ici MSC, pour mesenchymal stem cells) qui permettent la régénération de différents tissus tels que l'os ou le cartilage.

Les MSCs jouent un rôle crucial pour le tissu osseux car celui-ci est en constant renouvellement. L'os est constamment résorbé par les ostéoclastes et formé par les ostéoblastes. Ce phénomène, appelé remodelage osseux, joue un rôle important dans le maintien de la masse osseuse (Figure 16). Un déséquilibre entre la résorption et la formation osseuse peut être dû à une trop grande prolifération des ostéoclastes, ou à une mauvaise différenciation des MSCs en ostéoblastes.

Une accélération du remodelage osseux conduit à une ostéoporose, caractérisée par une diminution de la masse osseuse. Cette pathologie induit une augmentation du risque de fracture.

La différenciation de cellules souches en ostéoblastes, appelée ostéoblastogenèse, est contrôlée par plusieurs signaux, dont des signaux biochimiques enchâssés dans la MEC de l'os, et des signaux mécaniques. La tension sur l'os, transmise à la MEC, est ressentie par les longues protubérances des cellules mécanosensibles enchassées dans le tissu minéralisé : les ostéocytes. Grâce à ces informations, les octéocytes régulent l'équilibre résorption/formation du tissu osseux. C'est pourquoi une tension sur l'os est nécessaire à son bon développement.

C'est aussi l'une des pistes de recherche pour soigner l'ostéoporose : freiner la perte de densité osseuse en appliquant des tensions sur le squelette, par de l'exercice physique par exemple.



Figure 16 : L'équilibre résorption/formation du remodelage osseux. L'os, en constant remodelage, est à la fois résorbé par les ostéoclastes et formé par les ostéoblastes. Un déséquilibre de cette balance peut induire des processus pathologiques liés à une trop faible ou une trop forte masse osseuse. Adapté de (Sanchez-Duffhues et al., 2015)

4.2. Les facteurs de transcription impliqués dans l'ostéoblastogenèse

Comme mentionné précédemment, les cellules souches permettant la régénération de l'os peuvent venir de la moelle osseuse. Cependant, Colnot a mis en évidence une autre source potentielle, *in vivo*, de cellules souches dans les tissus avoisinants l'os : le périoste et l'endoste, permettant la régénération du cartilage et de l'os, respectivement (Colnot, 2009).

La cellule pré-ostéoblastique synthétise le collagène I et la BSP. Sous l'action de différents facteurs de transcription, notamment Runx2 (Runt-related transcription factor-2) (Ducy, 2000) et Ostérix (OSX), cette cellule se différencie en un ostéoblaste mature qui synthétise du collagène I, de l'OCN et de la phosphatase alcaline (ALP) permettant de minéraliser la MEC. D'autres facteurs de transcription interviennent lors de cette différenciation : Msx2 (msh homeobox homolog-2) et Dlx5 (Distal-less homeobox-5), qui activent l'expression de Runx2 et d'autres gènes plus tardifs comme l'OCN (Lee et al., 2005; Ryoo et al., 1997). La séquence des rôles de ces différents marqueurs de transcription est présentée Figure 17.



Figure 17 : Représentation schématique de la séquence des facteurs de transcription et de production de protéines de la matrice durant l'ostéoblastogenèse. Sous l'effet de Runx2, Dlx5 et Msx2, la cellule souche mésenchymateuse se différencie en pré-ostéoblaste. Sous l'effet de Runx2 et d'Ostérix, le pré-ostéoblaste se différencie en ostéoblaste. Concernant la production des protéines de la matrice, le pré-ostéoblaste produit le collagène I et la BSP tandis que l'ostéoblaste produit le collagène I, l'OCN et l'ALP.

Afin de maintenir la balance résorption/formation, les ostéoblastes favorisent la différenciation des cellules souches en ostéoclastes *via* l'activation de la voie RANK-L/RANK/OPG. Les ostéoblastes expriment la protéine RANK-L qui est reconnue par les précurseurs des ostéoclastes grâce aux récepteurs RANK, et cela conduit à la maturation des ostéoclastes.

4.3. Rôle et adaptation du système adhésif dans la différenciation osseuse 4.3.a. Les cadhérines et la différenciation osseuse

La condensation cellulaire est une étape nécessaire lors de la formation du tissu osseux (Hall and Miyake, 1995). C'est pourquoi le rôle des cadhérines lors de l'ostéoblastogenèse est très étudié.

Les cadhérines 11 et N sont cruciales pour la formation osseuse aussi bien *in vivo* (Di Benedetto et al., 2010) qu'*in vitro* (Kii et al., 2004). Cependant, Kawaguchi *et al.* ont montré que, contrairement à la N-cad qui est présente dans de nombreuses lignées mésenchymateuses, la cad-11 est spécifique aux lignées pré-ostéoblastiques et pré-adipocytaires (Kawaguchi et al., 2001), ce qui suggère des rôles différents. La N-cad est impliquée dans le maintien du pool de cellules stromales mésenchymateuses, tandis que la cad-11 favorise l'ostéogenèse au dépend de l'adipogenèse, mais n'est pas indispensable au développement post-natal de l'os. De plus, la N-cad étant cruciale pour les contacts

cellulaires, son inhibition affecte davantage la condensation cellulaire en nodules osseux (Di Benedetto et al., 2010).

4.3.b. Les intégrines et la différenciation osseuse

L'utilisation de biomatériaux étant une approche prometteuse pour la régénération osseuse, notamment les matériaux à base de protéine de matrice osseuse telle que le collagène, les interactions intégrine/MEC sont davantage étudiées dans la différenciation osseuse que dans la différenciation musculaire. Plusieurs intégrines ont un rôle dans la différenciation osseuse (Table 3). Tout d'abord, les intégrines ont un rôle sur la mécanotransduction, qui est un processus impliqué dans l'ostéogenèse. L'intégrine α 5 β 1 est une intégrine sensible à la rigidité de la MEC et elle permet, en retour, de réguler la tension du cytosquelette et, *in fine*, l'ostéogenèse (Brunner et al., 2011; Lee et al., 2015; Marie et al., 2014). De plus, une augmentation de l'expression d' α 5 dans des hMSCs induit une différenciation ostéoblastique (Hamidouche et al., 2009). L'intégrine β 5 semble aussi être impliquée dans l'ostéogenèse induite par une contrainte mécanique. Son expression est en effet fortement augmentée lors de l'ostéoblastogenèse de cellules souches mésenchymateuses dérivées du périoste du rat soumises à des pressions négatives (Zhu et al., 2014) et de cellules souches mésenchymateuses humaines dérivées de la moelle osseuse sous pression hydrostatique (Huang and Ogawa, 2012).

Intégrine	Ligand	Effets physiologiques	Références
α1β1	Collagène I	Adhésion cellulaire, différenciation	(Popov et al., 2011)
α2β1	Collagène I	Adhésion cellulaire, différenciation	(Popov et al., 2011; Shih et al., 2011)
α4β1	Fibronectine	Adhésion cellulaire, différenciation et formation osseuse	(Yao et al., 2013)
α5β1	Fibronectine	Adhésion cellulaire, différenciation, formation osseuse, mécanotransduction, régénération	(Batra et al., 2014; Brunner et al., 2011; Fromigué et al., 2012; Hamidouche et al., 2009; Watabe et al., 2011)
α11β1	Collagène I	Adhésion cellulaire	(Popov et al., 2011)
αVβ1	Ostéopontine	Différenciation	(Chen et al., 2014)
αVβ3	Fibronectine, sialoprotéine osseuse, vitronectine, ostéopontine	Adhésion cellulaire, différenciation, mécanotransduction	(Bonnet et al., 2012; Long et al., 2011; Su et al., 2010)

 Table 3 : Rôles des principales intégrines impliquées dans la différenciation osseuse.

 Adapté de (Marie, 2013; Marie et al., 2014)

Le rôle des intégrines dans l'ostéogenèse n'est pas lié uniquement à la mécanotransduction. Par exemple, l'intégrine $\alpha V\beta 1$, qui colocalise et co-immunoprécipite avec les BMPrécepteurs, a un rôle sur la réponse des cellules à la BMP-2 (Lai and Cheng, 2004). *In vitro*, la diminution des marqueurs osseux en réponse à la BMP-2 suite à l'utilisation d'anticorps bloquants contre $\alpha 1$, $\alpha 2$ et collagène I, révèle que la reconnaissance de la nature

4.3.c. Jeux croisés intégrines/cadhérines dans la différenciation osseuse

de la MEC par les intégrines est cruciale dans la réponse à la BMP-2.

Dans le cas spécifique de la différenciation osseuse, les systèmes adhésifs collaborent indirectement *via* différentes voies de signalisation, impliquant par exemple les kinases ou Wnt/β-catenine.

L'ostéogenèse étant influencée par la mécanotransduction, les jeux croisés des intégrines et des cadhérines sur la tension du cytosquelette permettent de réguler indirectement la différenciation osseuse (Marie et al., 2014).

La voie Wnt/ β -catenine est aussi régulée à la fois par les cadhérines -la N-cad séquestrant la β -catenine à la membrane, ce qui inhibe sa translocation au noyau et par conséquent son rôle de transcription- et par les intégrines -l'activation de FAK et de ILK par les intégrines inhibant la dégradation de la β -catenine par GSK3 β (Figure 18 (Marie et al., 2014)).



Figure 18 : Les interactions indirectes entre intégrines et cadhérines via la voie Wnt/ β -catenine. La liaison intégrine/ligand induit le recrutement et l'activation de ILK, ce qui entraîne l'inhibition de GSK3, qui ne peut donc plus dégrader la β -catenine. La β -catenine ne peut pas alors se transloquer au noyau et activer des gènes ostéogéniques. Grâce à son domaine cytoplasmique, N-cad (CDH2) interagit aussi avec la β -catenine mais pour la séquestrer à la membrane et donc empêcher sa translocation au noyau. Adapté de (Marie et al., 2014)

De plus, la récente découverte des interactions de cad-11 et de l'intégrine β 1 aux plaques focales d'adhésion (Langhe et al., 2016) pourrait suggérer aussi une coopération directe dans l'ostéogenèse, cad-11 étant la cadhérine des tissus osseux.

5. COMMUNICATION INTER-ORGANES ENTRE LE MUSCLE ET L'OS

Il est important de noter que les tissus osseux et musculaire ont la même origine embryologique, dérivant tout deux des somites (Lemos et al., 2015).

5.1. Coopération mécanique et biochimique entre tissu musculaire et tissu osseux

5.1.a. Une interaction mécanique entre le muscle et l'os

Des phénomènes observés en clinique ont amené à se pencher sur les contributions muscle/os lors de la régénération de ces deux tissus. Les cliniciens ont constaté que les fractures déplacées se réparent moins bien que les fractures non déplacées. Les fractures déplacées sont caractérisées par une déchirure des tissus mous avoisinant l'os, comme le muscle.

Des études plus poussées sur le sujet ont aussi montré que des embryons de poulet et de souris paralysés, donc sans tension générée par le muscle, présentaient des problèmes de formation des cartilages et des os (Shwartz et al., 2013). Ceci met en évidence une première contribution mécanique entre les muscles et les os. En effet, comme nous l'avons vu, les os ont besoin d'une certaine tension pour se former correctement. Le stress mécanique affecte l'élongation des cartilages et la production de la MEC (Berendsen and Olsen, 2015). En conditions physiologiques, les muscles contribuent à maintenir la tension suffisante pour le bon développement de l'os.

Les mécanismes cellulaires amenant aux coopérations mécaniques font l'objet de nombreuses études. Du côté du muscle, mTORC1 (*mammalian target of rapamycin complex 1*) est le médiateur central de la réponse du muscle à un stimulus mécanique. mTORC1 est une enzyme de la famille des sérine/thréonine kinases qui régule différentes signalisations cellulaires telles que la prolifération ou la transcription, et qui est impliquée dans certaines myopathies (Risson et al., 2009). Cependant, les voies de signalisation amenant à son activation en réponse à un stress mécanique ne sont pas encore complétement élucidées. Différentes protéines sont suspectées d'y contribuer, une des plus probables étant PA (*Phosphatidic acid*) qui contribue à l'activation de mTORC1 en s'y liant (You et al., 2012).

Du côté de l'os, c'est la voie Wnt qui est le médiateur central de la réponse à un stimulus mécanique. Les ostéocytes, connus pour être les chefs d'orchestre du remodelage osseux et particulièrement en réponse à un stress mécanique, montrent une augmentation de l'activité transcriptionnelle de la β -catenin en réponse à une contrainte mécanique (Goodman et al., 2015).

Cependant, les interactions muscle/os sont bien plus complexes que de simples interactions mécaniques. Par exemple, l'apposition de lambeaux musculaires sur des fractures ouvertes améliore grandement la régénération osseuse (Harry et al., 2008). En fait, ces deux tissus partagent aussi de nombreux signaux biochimiques permettant à chacun d'aider à la régénération de l'autre (Brotto and Bonewald, 2015).

5.1.b. Les jeux croisés biochimiques entre le muscle et l'os

D'un côté, les cellules musculaires sécrètent différentes molécules endocrines qui aident à la régénération osseuse. Cette aide peut être indirecte : les cellules musculaires sécrètent des facteurs comme les interleukines qui favorisent la vascularisation de l'os et *in fine* sa régénération (Quinn et al., 2009) ; ou directe, en sécrétant aussi des signaux biochimiques qui bénéficient directement aux cellules osseuses. Il a été récemment montré que la myokine Irisin, sécrétée par les cellules des muscles squelettiques après un effort physique, affecte directement le métabolisme osseux (Colaianni et al., 2014, 2016). *In vitro*, des facteurs sécrétés par les myotubes, et non les myoblastes, augmente la viabilité d'ostéocytes. Le rôle protecteur des myotubes sur les ostéocytes a été validé *in vivo*, et uniquement lorsque les muscles étaient stimulés électriquement (Jähn et al., 2012).

La première preuve d'une contribution directe des cellules musculaires à la régénération osseuse a été apportée en 2015 par Abou-Khalil *et al.* (Abou-Khalil et al., 2015). Les auteurs ont montré que les cellules satellites permettent l'activation des cellules du périoste par la production de facteurs de croissance osseux tels que les BMPs.

D'un autre côté, les cellules osseuses sécrètent des signaux biochimiques favorisant la régénération musculaire. C'est le cas de l'ostéocalcine, qui est produite par les ostéoblastes,

et qui permet non seulement la minéralisation de la matrice osseuse mais a aussi un effet sur l'activité musculaire (Karsenty et al., 2016; Levinger et al., 2014).

Les tissus musculaires et osseux communiquent aussi en partageant des voies de signalisation. La voie Wnt/βcatenin, impliquée dans l'ostéogenèse ainsi que dans la réponse du tissu osseux à une contrainte mécanique, est une de ces voies communes. En effet, une fois transloquée au noyau, la β-catenin active non seulement des gènes clefs dans l'ostéogenèse en se liant à Lef1/TCF, mais aussi des gènes clefs dans la myogenèse en formant des complexes avec des facteurs de transcription de la myogenèse tels que myoD et myogenin (Kim et al., 2008). Une contrainte mécanique sur le tissu osseux favoriserait donc la régénération musculaire *via* cette voie. Le rôle de la voie Wnt dans les interactions muscle/os a fait l'objet d'une revue (Rudnicki and Williams, 2015) soulignant notamment la communication entre ces deux tissus *via* la voie non canonique de Wnt, impliquant les rhoGTPases.

Enfin, la dédifférenciation des myotubes sous Msx2 évoquée section 3.2, permettant aux myotubes de se cellulariser et de reprendre leur prolifération, est directement influencée par le tissu osseux. En effet, Msx2 est un facteur de transcription qui est exprimée dans la réponse au facteur de croissance ostéoinducteur BMP-2. La régénération du tissu musculaire est donc meilleure quand la BMP-2 est proche.

De plus, les BMPs permettent de réguler l'équilibre prolifération/différenciation des cellules satellites. Une présentation contrôlée de la BMP aux cellules satellites permet d'augmenter leur prolifération tout en évitant une différenciation myogénique trop précoce. En effet, en réponse à la BMP-2, le gène inhibiteur de la myogénèse, Id1, est exprimée. Ce qui freine la différenciation des cellules satellites, au bénéfice de leur prolifération. Ensuite, lorsqu'elles entrent en différenciation myogénique, les cellules satellites sécrètent la protéine Noggin, un antagoniste de la BMP. L'effet inhibiteur de la BMP sur la différenciation myogénique est ainsi stoppé. Dosée convenablement, les BMPs permettent donc, par le biais de la prolifération des cellules satellites, de favoriser la régénération musculaire (Ono et al., 2011).

Au-delà des facteurs biochimiques échangés, une contribution cellulaire est aussi mise en avant. L'application de lambeaux musculaires n'aide pas uniquement à la vascularisation de l'os, mais sert également de second périoste capable de fournir des cellules souches à l'os (Liu et al., 2010; Schindeler et al., 2009).

5.2. Le muscle comme source de cellules souches pour la régénération osseuse 5.2.a. Contribution des cellules souches musculaires pour la régénération osseuse

Les formations ectopiques d'os lors de processus pathologiques tels que la Fibrodysplasie Ossifiante Progressive (FOP) révèlent le potentiel ostéogénique du tissu musculaire (Shafritz and Shore, 1996). Cette pathologie, aussi appelée la maladie des hommes de pierre, se caractérise par l'ossification progressive des muscles squelettiques, c'est-à-dire une transdifférenciation des cellules musculaires squelettiques (Figure 19) en cellules osseuses. Progressivement, un deuxième squelette se forme et tout mouvement devient impossible. Cette maladie orpheline, dont la cause génétique sera détaillée dans la section suivante (Shore et al., 2006) concerne un peu plus d'une naissance par million et diminue l'espérance de vie à 40 ans. Outre cette pathologie critique, la formation osseuse hétérotopique peut survenir spontanément ou suite à une opération telle que l'arthroplastie totale de la hanche. Les conséquences sont plus ou moins graves selon sa localisation et, dans les cas les plus critiques, peuvent nécessiter la chirurgie.

En plus de révéler le potentiel ostéogénique des tissus musculaires, ces processus pathologiques illustrent l'importance de comprendre les mécanismes cellulaires intervenant dans la transdifférenciation des cellules musculaires vers les cellules osseuses.



Figure 19 : Illustration des effets de la Fibrodysplasie Ossifiante Progressive. La posture rigide de cet homme de 25 ans atteint de Fibrodysplasie Ossifiante Progressive était due à la fusion de ses os au niveau de la colonne vertébrale, des épaules et des coudes. (Shafritz and Shore, 1996)

Schindeler *et al.* suggèrent que, lorsque les cellules du périoste ou de la moelle osseuse ne suffisent pas, les cellules venant des tissus voisins, particulièrement les cellules musculaires, peuvent jouer un rôle important dans les fractures osseuses (Davis et al., 2015; Schindeler et al., 2009). En effet, comparé à d'autres types cellulaires tels que les cellules stromales primaires, les chondrocytes articulaires primaires et les fibroblastes primaires, les cellules dérivées du muscle exprimant BMP-2 sont celles qui produisent le plus de matrice quand elles sont implantées dans les membres postérieurs des souris (Musgrave et al., 2000). De plus, l'expression de myoD dans les fibroblastes C3H10T1/2 augmente leur réponse à un traitement BMP, ce qui indique que leur capacité à se différencier en cellules musculaires favorise leur capacité à se différencier en cellules osseuses (Komaki et al., 2004).

Liu *et al.* ont montré qu'une lignée myogénique pouvait participer à la réparation d'un défaut osseux lors d'une fracture ouverte. Après l'insertion de ces cellules myogéniques dans un tel défaut osseux, elles ont présenté une morphologie chondrogénique et ont fini enchâssées dans la matrice osseuse, suggérant un rôle fonctionnel lors de la régénération tissulaire (Liu et al., 2011b).

Les mécanismes contrôlant, *in vivo*, la décision des cellules musculaires de se différencier vers l'os ne sont pas encore complétement élucidés mais sont probablement contrôlés par le tissu osseux. Contrairement à la section 5.1, nous allons maintenant détailler une voie de signalisation permettant aux cellules osseuses de recruter et contrôler les cellules musculaires pour les aider à réparer le tissu osseux.

5.2.b. Effets de la BMP-2 sur les cellules musculaires i. La transdifférenciation des cellules musculaires sous BMP-2

Comme expliqué précédemment, la BMP-2 est connue pour inhiber la myogenèse *via* le facteur de transcription Id1. En fait, ce facteur de croissance ostéogénique permet non seulement d'inhiber la myogenèse des précurseurs musculaires, mais aussi d'induire leur ostéogenèse. En effet, la BMP-2 est reconnue par les BMP récepteurs aux membranes des cellules, ce qui entraîne l'ostéogenèse *via* la voie SMAD et non-SMAD (voir partie 6, Figure 2).

Campbell *et al.* utilisent une population de cellules souches primaires dérivées du muscle de souris adulte (MDSCs) pour valider le potentiel ostéogénique de leurs micro-motifs de BMP-2 (voir section 6.3.b.ii) ; celles-ci répondant positivement pour l'ALP sur les motifs, et fusionnant en myotubes en dehors des motifs (Phillippi et al., 2008).

C'est justement ce processus qui est mis en cause dans la FOP. En conditions physiologiques, la voie BMP-2 est activée lorsque les BMP-récepteurs à la membrane cellulaire reconnaissent la BMP-2. Chez les personnes atteintes de FOP, une mutation génétique spontanée a engendré la modification d'un de ces récepteurs, qui est alors constamment actif (Shore et al., 2006). Lors d'une inflammation suivant un traumatisme, même léger, des cytokines sont produites afin d'augmenter la prolifération cellulaire. Les cellules, sensibilisées par les cytokines et ayant un récepteur défectueux dû à la mutation, agissent alors comme si elles étaient constamment sous BMP-2 et s'engagent en différenciation osseuse. C'est pourquoi la moindre inflammation entraîne une ossification des tissus musculaires.

Le fait que les cellules musculaires se différencient en cellules osseuses sous BMP-2 remet en perspective ce qui a été vu sur la cellularisation des myotubes (section 3.2). Nous avons vu que sous BMP-2, les cellules osseuses produisent le facteur de transcription Msx2 qui permet la dédifférenciation des myotubes. Grâce à la BMP-2, ces myotubes dédifférenciés peuvent alors servir de réservoir de cellules souches non seulement pour la régénération musculaire mais aussi pour la régénération osseuse.

Le potentiel de la BMP-2 est donc énorme pour la régénération des tissus osseux et musculaires. Selon la concentration de la BMP-2 et la phase à laquelle elle est présentée aux cellules, cette protéine permettrait à la fois :

- de dédifférencier des myotubes pour refaire un stock de cellules souches ;
- d'aider la prolifération des cellules satellites en inhibant une myogenèse trop précoce ;
- et d'induire la différenciation osseuse de cellules souches mésenchymateuses ou de cellules souches dérivées du muscle.

De plus, un récepteur BMP étant impliqué dans des processus pathologiques tels que FOP, cela souligne l'intérêt de comprendre les mécanismes cellulaires impliqués dans la réponse à la BMP-2.

ii. Le modèle C2C12

Les cellules C2C12 sont une lignée de myoblastes murins (Blau et al., 1983) pouvant se trandifférencier vers une lignée ostéogénique sous l'effet de la BMP-2 (Katagiri et al., 1994a). En effet, en présence de BMP-2, la myogenèse des cellules C2C12, caractérisée par la fusion des cellules en myotubes et l'expression de la troponine T, est complétement inhibée. De plus, les cellules sécrètent de l'ostéocalcine et sont positives pour l'ALP (Figure 20).



Figure 20 : Effet de la BMP-2 sur les C2C12. Les cellules C2C12 sont cultivées sans (a et c) ou avec (b et d) de la BMP-2 en solution (à 300 ng/mL). Après 6 jours de culture, les marqueurs myogénique troponinT (a et b) et ostéogénique ALP (c et d) sont marqués. (Katagiri et al., 1994a)

Comme illustré sur la Figure 20, ces cellules présentent l'avantage d'être complétement négatives pour l'ALP en l'absence de BMP-2. L'utilisation des cellules C2C12 permet donc d'étudier spécifiquement l'effet de la BMP-2 et comment celle-ci déclenche l'ostéogenèse. C'est pourquoi elles sont largement utilisées comme modèle depuis les premiers travaux de Katagiri (Katagiri et al., 1994a). Le grand intérêt que suscitent ces cellules a permis le développement d'outils tels que les C2C12 A5, aussi appelée C2C12 BRE-Luc permettant l'expression d'un gène reporter luciférase sous le contrôle du promoteur Id1. Pour obtenir cette lignée cellulaire, les C2C12 classiques ont été transfectées avec le gène codant pour la luciférase (d'où « Luc ») associé au promoteur d'Id1, répondant aux BMP-2, 4 et 7 (d'où « BRE », pour BMP Responsive Element) (Logeart-Avramoglou et al., 2006). En présence

de BMP-2, ces cellules sécrètent la luciférase qui peut être détectée par des kits commerciaux classiques. En utilisant ces cellules, la mesure de la réponse à la BMP-2 est plus rapide et 100 fois plus sensible qu'une détection de l'ALP.

Sous BMP-2, les C2C12 adaptent leur répertoire de cadhérines lors de cette transdifférenciation muscle/os. Alors que M-cad est la cadhérine majoritairement exprimée par les myoblastes C2C12, son expression est inhibée par la BMP-2, au profit de cad-11. N-cad est aussi exprimée par les C2C12 mais son expression est beaucoup moins affectée que les autres par la différenciation ostéogénique ou musculaire (Kawaguchi et al., 2001; Shin et al., 2000) (Figure 21).



Figure 21 : Représentation schématique du changement de répertoire des cadhérines chez les C2C12 sous BMP-2. Initialement, R, M et N-cad sont présents. Lorsque les cellules se différencient en myotubes, R-cad cesse d'être exprimé tandis que M et N-cad sont exprimées tout au long de la myogenèse. Sous BMP-2, l'expression de R-cad et M-cad diminue tandis que cad-11 est présente. Synthèse à partir de (Kawaguchi et al., 2001; Shin et al., 2000)

Comme mentionné en section Chapitre I.5.2.b.i, la BMP-2 est certes un facteur de croissance prometteur pour la régénération osseuse, mais pour bénéficier au mieux de ses propriétés il est crucial de comprendre les mécanismes cellulaires impliqués en réponse à cette protéine. *In vivo*, la BMP-2 est présentée enchâssée dans la MEC. Or, comme nous l'avons vu, les intégrines, récepteurs de la MEC, sont impliquées dans la différenciation cellulaire. Pour étudier la réponse à la BMP-2, il convient donc de reproduire le microenvironnement cellulaire, c'est-à-dire la présentation par la matrice de la BMP-2.

Pour cela, différents biomatériaux ont été développés ces dernières années afin de présenter la BMP-2 par la matrice pour les études *in vitro*. Comme nous allons le voir, ces biomatériaux permettent d'étudier différents aspects de la réponse à la BMP-2.

6. TUNING CELLULAR RESPONSES TO BMP-2 WITH MATERIAL SURFACES – *revue*

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ABSTRACT:

Bone morphogenetic protein 2 (BMP-2) has been known for decades as a strong osteoinductive factor and for clinical applications is combined solely with collagen as carrier material. The growing concerns regarding side effects and the importance of BMP-2 in several developmental and physiological processes have raised the need to improve the design of materials by controlling BMP-2 presentation. Inspired by the natural cell environment, new material surfaces have been engineered and tailored to provide both physical and chemical cues that regulate BMP-2 activity. Here we describe surfaces designed to present BMP-2 to cells in a spatially and temporally controlled manner. This is achieved by trapping BMP-2 using physicochemical interactions, either covalently grafted or combined with other extracellular matrix components. In the near future, we anticipate that

material science and biology will integrate and further develop tools for *in vitro* studies and potentially bring some of them toward *in vivo* applications.

KEYWORDS: BMP-2, material surface, cell adhesion, growth factor immobilization, BMP receptors, signaling

6.1. Introduction

Bone morphogenetic protein-2 (BMP-2) is a multifunctional growth factor belonging to the transforming growth factor- β (TGF- β) superfamily. It was identified in the 1970s as an essential molecule for de novo bone formation in adult animals (Urist, 1965; Urist et al., 1973). Indeed BMP-2 is one of the strongest osteoinductive factors known so far: it initiates the differentiation of mesenchymal stem cells (MSCs) into osteoblasts and chondrocytes in vivo and in vitro (Ryoo et al., 2006), as well as the transdifferentiation of muscle cells into bone cells (Asakura et al., 2001; Katagiri et al., 1994).

In view of its osteogenic potential, the clinical use of recombinant human BMP-2 (rhBMP-2), first purified in 1988 by Wang *et al.* (Wang et al., 1988), was approved in 2002 by the Food and Drug Administration (FDA) and validated by the European Medicines Agencies. To date, the only FDA-approved material carrier is an absorbable collagen sponge to which a high amount of rhBMP-2 is applied (up to 2.1 mg/level) (Geiger et al., 2003), due to its poor affinity for collagen (Kim and Valentini, 2002). In clinical trials, it has been reported that up to 23% of patients suffered complications, such as hematomas and swelling in the neck and throat regions (Carragee et al., 2011), dysphagia and a heightened risk of cancer (Fu et al., 2013). In Europe, while the clinical use of rhBMP-2 as an adjunct to standard care has been approved, the increasing number adverse event reports and the growing socio-economic need for bone repair therapies raise the important question of how to develop effective materials which allow the control of the biological responses to BMP-2.

In the last decade, several studies have shown the possibility to deliver BMP-2 from various carrier materials (King and Krebsbach, 2012; Lo et al., 2012; Schmidmaier et al., 2008) especially polymeric materials and ceramics. Since *in vitro* tests were promising and preclinical studies are currently being performed, it is likely that future medical devices containing new formulations of BMP-2 will be approved. However, it is still challenging to achieve a controlled presentation of BMP-2, while retaining its activity and minimizing the amount of protein applied locally. Standard biological studies stimulate cells with BMP-2 added to the culture media. In these cases, high amounts of the growth factor are needed because of the limited lifetime of BMP-2 in solution. Additionally, this condition does not represent the natural cellular environment, since BMP-2, like other growth factors, is sequestered in the extracellular matrix (ECM) and released upon matrix degradation (Frenkel et al., 2000; Ruppert et al., 1996). Thus advanced biomaterials which take into consideration the physical and chemical complexity of the extracellular environmental are being developed. These materials could serve as a tool for biologists to unravel novel biological properties of BMP-2 which could not be explored so far with standard culture methods (Katagiri et al., 1994a).

A timeline showing a few of the most important findings on BMP-2 in biological and material sciences is shown in Figure 1. It is noteworthy that approval for the *in vivo* use of BMP-2 (2003) took place before the development of advanced materials able to control and reduce BMP-2 release and before the discovery of new biological functions of BMP-2 such as its influence on the whole human body. Hence, there is now a great need to build an integrative approach including material science, chemistry, engineering, biochemistry and cell biology, and bridge the gap between these different disciplines. From the materials side, researchers could bring innovations in the design of materials for BMP-2 presentation by providing functionalization strategies and characterization methods as well as by developing new tools for the spatial control of BMP-2 delivery using micro- and nanotechnology approaches. From the biochemical and biological standpoint, researcher could provide new tools to produce BMP proteins, engineered mutants or tagged molecules.

In this review, we first summarize the emerging functions of BMP-2 in cell biology and the resulting signaling responses at the interface between cells and their environment. We then present recent developments on engineered surfaces that aim at mimicking the presentation of BMP-2 in its natural environment. Finally, we discuss how specific properties of materials may help in optimizing existing systems or may bring new ideas for the design of innovative delivery systems.



Figure 1: Time-line showing few of the most important findings on BMP-2 in biology (in pink) and in material sciences (in blue). Fundamental biological discoveries such as the influence of BMP-2 in the whole human body and the development of advanced materials able to modulate the physicochemical presentation of BMP-2 followed the approval for the in vivo use of BMP-2 in 2002.

6.2. Cell responses to soluble BMP-2

Although BMP-2 signaling has historically been linked to bone, the growing number of known BMPs functions in different tissues brought the biology community to coin a new term for all bone morphogenetic proteins: "body morphogenetic proteins" (Reddi et al., 2005). Figure 2 schematically illustrates the major steps for the BMP-mediated induction of osteogenic differentiation in bone progenitor cells and myoblasts, which transdifferentiate into osteoblasts upon BMP-2 stimulation (Katagiri et al., 1994). BMP-2, like other members of the TGF- β superfamily, signals upon binding to two types of cell transmembrane serine/threonine kinase receptors, the BMP type I (BMPRI) and type II (BMPRII) receptors. The binding of BMP-2 to BMPRI results in the phosphorylation of SMAD1/5/8, which forms a complex with co-SMAD (SMAD4) and translocates to the nucleus (Liu et al., 1996). For transcriptional signaling, this shuttling leads to a subsequent expression of transcription factors such as Id-1 and BMP-2 responsive element, typical markers of osteogenic differentiation (Katagiri et al., 2002). At later time points, alkaline phosphatase (ALP) is expressed after several days, and mineralized matrix deposition is detected after several weeks of culture (Ryoo et al., 2006). Besides the SMAD pathway, gene transcription is induced by BMP-2 via non-SMAD signaling as BMP induces the MAPK pathway, which leads to the expression of ALP, osteopontin and collagen I (for details about signaling, see

review (Sieber et al., 2009)). Regarding the non-transcriptional signaling mediated by BMP-2, recent studies have shown that BMPRs might control cytoskeletal rearrangements involved in cell migration (Gamell et al., 2008, 2011; Hiepen et al., 2014; LInd et al., 1996). The regulation of BMP signaling takes place at several levels, from receptor complex formation to crosstalk with other pathways, as will be discussed in the next paragraphs.



Figure 2: Schematic representation of the major steps in the differentiation of bone progenitor cells and myoblasts over time. Note that the crosstalk with other signaling pathways and other relevant markers are omitted for simplicity. The relative size of all molecules is not drawn to scale.

6.2.a. Modulation of BMP-2 signaling at the cell surface

i. BMP receptor complex formation

Studies from P. Knaus group have indicated that BMP receptors present a distinct mode of oligomerization and activation (Nohe et al., 2002). For the formation of a functional signaling receptor complex, BMP-2 binds to BMPRI, which is either already organized in a receptor complex with BMPRII, or recruits BMPRII. These modes of oligomerization result in the activation of different signaling pathways: binding of BMP-2 to a pre-formed complex induces the classical SMAD signaling pathway, while ligand-induced oligomerization induces the non-SMAD pathway. So far, these events have been analyzed by applying biochemical separation of detergent-resistant membranes and co-immunoprecipitation methods (Gilboa et al., 2000). There is still little information regarding the spatial arrangement of BMPRs at the nanoscale and the localization of the different complexes in distinct cellular compartments. Only recently was the spatial distribution of BMPRIb and

BMPRII visualized using high-resolution imaging techniques. Using two-color Stimulated Depleted Emission (STED) microscopy (Figure 3A), single BMPRII appear to arrange sparsely, whereas BMPRI assemble in larger clusters comprised of multiple receptors (Medda et al., 2016). When BMP-2 was added to the cell culture media, the BMPRII associated with the larger BMPRI assemblies at the cell periphery. The lateral mobility of BMPRI and BMPRII is also very distinct, as shown by single particle tracking experiments: in fact BMPRI is very confined, both in presence or absence of the ligand, whereas the mobility of BMPRII can be either confined or free diffusing (Guzman et al., 2012). The preformed complex, which triggers the SMAD-dependent pathway, does not require the confined movement of BMPRI, while the non-SMAD seems to be highly dependent on the localization of BMPRI in membrane microdomains. Thus, non-SMAD signaling might require more stable complexes, possibly to allow interaction with other protein complexes, e. g. those involved in signaling to the cytoskeleton. To determine BMPR localization, the successful expression of tagged receptors has been possible for overexpression of human influenza hemagglutinin (HA)-tagged BMPRII (Marom et al., 2011) and it remains very challenging for BMPRI because of its low expression level. Tools are currently lacking in order to combine high-resolution approaches with studies on the dynamics of receptor complex formation and to identify the physical determinants of receptor mobility.



Figure 3: A, Confocal microscopy (right) and STED microscopy (left) images of BMPRIb (in green) and BMPRII (in red). In the absence of BMP-2, the two different receptors rarely co-localized (upper white arrowhead) and BMPRII did not cluster (lower arrowhead). When cells were exposed to BMP-2, BMPRII associated with the larger BMPRIb assemblies. This different behavior could not be appreciated with confocal microscopy. Image adapted from (Medda et al., 2016). B, Example of colocalization (indicated by arrows) of BMPRI and BMPRII (red) with avß5 integrins (green) detected by confocal microscopy. Images adapted from (Lai and Cheng, 2005).

ii. Receptor-ligand internalization

For BMP-mediated signaling the receptor complexes are internalized in two possible ways: (i) caveolae pits are formed for BMPRI and recruited BMPRII complex and activate non-SMAD pathways; (ii) clathrin-dependent internalization is required for the preformed receptor complex resulting in the activation of the SMAD pathway (Hartung et al., 2006). Different points of discussion have been raised regarding clathrin-dependent internalization of the ligand-receptor complex in growth factor signaling. The first point is whether receptor internalization is required for signaling. For tyrosine kinase receptors, such as vascular epidermal growth factor receptor 2 (VEGFR2) and epidermal growth factor receptor, clathrin-mediated endocytosis is important in regulating receptor recycling to modulate the amplitude of biological response (Vieira et al., 1996). VEGFR2 internalization is required for the activation of ERK1/2 signaling but dispensable for other signaling pathways (Gourlaouen et al., 2013). For serine/threonine kinase receptors, such as BMPRs, recent studies combining confocal and atomic force microscopy (AFM) have indicated that BMP-2 signaling might already start in domains of the plasma membrane outside of clathrin-coated pits, where BMP-2 molecules bind to BMPRIa, which then phosphorylates and triggers SMAD signaling (Bonor et al., 2012). Regarding downstream signaling events, the treatment of cells with endocytosis inhibitors does not affect SMAD phosphorylation, while the downstream signal propagation is hindered (Chen, 2009; Hartung et al., 2006; Jortikka et al., 1997). Conversely, inhibition of BMP-2 endocytosis by an epigenetic approach actually elevates transcriptional responses (Rauch et al., 2002). Additionally, dynamin inhibition impairs osteogenic differentiation but does not block completely the transcriptional activation of several other genes, suggesting the presence of alternative SMAD-dependent signaling cascades which are independent of endocytosis (Heining et al., 2011).

These biochemical approaches to inhibit endocytosis lead to another point of discussion related to growth factor internalization. As of today, it remains elusive whether the ligand has to remain bound to the receptors and become internalized *via* the clathrin-mediated pathway, or if it would be sufficient to have trafficking of the activated receptors, regardless of ligand internalization. In 1997 Jortikka *et al.* (Jortikka et al., 1997) reported that bonds with carrier materials should not be tight nor in covalent form to allow endocytosis of BMP-2. However, recent studies demonstrated that anchorage of the growth factor to the ECM or to a surface still conveys signaling by prolonged activation of receptors and differential phosphorylation (Pohl et al., 2012; Schwab et al., 2015). Thus, ligand-receptor interaction at

the cell membrane might be sufficient to obtain a sustained signaling response. It remains to be elucidated if a mechanical component causes deformation of the membrane and affects internalization signaling or if co-recruitment of other adhesion receptors such as integrins might occur in these cases where the BMP-2 molecules cannot be internalized.

6.2.b. BMP-2 signaling in a cell adhesion context

i. Osteogenic and adhesion signaling crosstalk

Extracellular factors orchestrate the commitment and differentiation of many cell types; in turn, a concerted action of adhesive and growth factor signals regulates adhesion and motility, which are mediated by interactions with the physical and biochemical cues from the environment. The signaling crosstalk between BMP-dependent and integrin-mediated pathways has been explored towards the modulation of both osteogenic differentiation and adhesion to the ECM (Kwon et al., 2013). Regarding the participation of integrin signaling in the transcription of genes for osteogenic differentiation, the collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ regulate BMP-induced differentiation by acting downstream of BMPRI (Jikko et al., 1999; Reyes and García, 2004). Moreover, following binding to collagen, FAK phosphorylation is necessary for the transcriptional activity of SMAD6 but not for the translocation of SMAD1 (Tamura et al., 2001). α_v integrin also regulates BMP-dependent osteogenic differentiation (Lai and Cheng, 2005), and in particular osteoblastic response to CYR61, a bone activator that increases the level of BMP-2 and activates the $\alpha_v\beta_3$ integrin/ILK/ERK signaling pathway (Su et al., 2010).

For the regulation of adhesion, as of today only few studies have shown the impact of BMP signaling on integrins and integrin-mediated structures. Lai *et al.* (Lai and Cheng, 2005) reported that during 4 days stimulation of osteoblasts with BMP-2 in the media, the expression of α_v integrins is increased, BMPRs colocalize with α_v and β_1 integrins in focal adhesions (Figure 3B) and coprecipitate with these receptors. However, the colocalization pattern with vinculin, a structural protein present in focal adhesions, could not be confirmed by recent studies using high-resolution microscopy (Medda et al., 2016). In osteoblasts, BMP-2 enhances the formation of focal adhesions and stress fibers by increasing α_5 and β_1 integrin expression, and triggers migration events by enhancing the incorporation of β_1 integrin into lipid rafts (Shah et al., 1999; Sotobori et al., 2006).

In this context, there are still several key questions that remain unanswered and might add further complexity to the entire picture encompassing BMP and adhesion signaling. First, it should be elucidated where binding sites for integrins and BMPR are located relative to each other within the extracellular matrix. As a consequence, there is the need for a deep understanding of how BMPRs and integrins are spatially organized at the plasma membrane to allow both physical interactions and signaling crosstalk. Finally, it should be determined how multiple pathways modulating adhesion dynamics are regulated spatio-temporally.

ii. Effects of BMP-2 on cytoskeleton assembly and cell migration

The evidence that BMP signaling is involved in the crosstalk with other pathways has brought to attention new functions of BMP-2, which are not necessarily related to its transcriptional signaling pathways. For example, BMP-2 signaling is involved in wound healing and cancer invasiveness by acting on actin cytoskeleton dynamics (Ehata et al., 2013; Moustakas and Heldin, 2008; Padua and Massagué, 2009). Upon BMP-binding to the BMPR complex, LIMK1 dissociates from BMPRII and phosphorylates cofilin (Foletta et al., 2003). The activation of LIMK1 by BMP-2 initiates the signaling to the cytoskeleton in a PI3K-dependent manner; a concomitant activity of Cdc42 is however required (Gamell et al., 2008).

Hiepen *et al.* (Hiepen et al., 2014) have recently shown that a regulatory subunit of PI3K is essential in directed cell migration mediated by BMP-2 at the leading edge of migrating cells. BMP-2 also induces the activation of the p38/MK2/Hsp25 pathway at cortical actin protrusions in migrating cells (Gamell et al., 2011). To further add complexity, other signaling pathways independent from LIMK1 activation have been identified, where actomyosin assembly is mediated by ROCK1 kinase downstream of Rho GTPases and myosin light chain kinase (Konstantinidis et al., 2011). Taken together, these studies clearly indicate that BMP-2 participates in the regulation of cell protrusion formation and migration, acting on multiple parallel pathways involved in actin reorganization. However, as for the interaction of the receptors at the plasma membrane, the spatio-temporal aspects of such regulation of signaling to the cytoskeleton still remain unclear.

These new and intriguing functions of BMP-2 are also relevant for the design of biomaterials/implants for the delivery of BMP-2, adhesion being the first step at the interface between cells and artificial materials. In turn, many answers to these open questions might come in the near future with the aid of material science approaches which allow control over the presentation of BMP-2 to cells.

6.3. Mimicking the BMP-2 microenvironment with material surfaces

Several growth factors are present in tissues in a matrix-bound form and released upon matrix degradation (Frenkel et al., 2000; Ruppert et al., 1996). The mode of presentation of BMP-2 at the interface with cells might be crucial in modulating its biological activity. For this reason, material surfaces applied to biological studies should mimic the physicochemical properties of the native ECM, to facilitate and allow predictions of cellular responses. In particular, using materials that enable the control of the amount of BMP-2 on their surface and its local distribution might help in determining the spatio-temporal regulation of BMP-2 signaling pathways.

In comparison with soluble BMP-2 (Figure 4A), the presentation of the growth factor on material surfaces could be tailored to achieve controlled immobilization and/or release of the protein from the surface (Figure 4B). This might lead to different signaling kinetics as well as the activation of alternative signaling pathways. Additionally, modifications in surface chemistry which allow the spatial control of BMP-2 (Figure 4C) could support the quantitative analysis of signaling events. Finally, surfaces where BMP-2 is presented together with ECM components (Figure 4D) could maintain or even enhance the biological activity of BMP-2 while possessing adhesive properties to allow the growth and colonization of cells.

6.3.a. Temporal control of BMP-2 activity with material surfaces

In the design of materials aiming at achieving a time-controlled presentation of BMP-2, the growth factor can be immobilized on surfaces either by physical entrapment (i. e. electrostatic interaction, hydrophobic effect, hydrogen-bonds) which allows a slow release and

internalization of the molecule, or by immobilization through a chemical linker or through biotin-Streptavidin (SAv) binding, which leads to a sustained presentation of BMP-2 (Figure 4B).



Figure 4: Schematic representation of different material surfaces approaches to control the presentation of BMP-2 at the interface with cells. A, BMP-2 is added to the cell culture media, which represents the standard stimulation way. B, BMP-2 either entrapped by electrostatic interactions (left) or chemically bound to the material surface (right). C, Surface patterning of BMP-2 for the spatial control of BMP-2 presentation. As an example, gradients of matrix-bound BMP-2 are schematically shown. D, Copresentation of BMP-2 and ECM components.

i. Physical entrapment of BMP-2

The formation of Layer-by-Layer (LbL) polyelectrolyte multilayer films is a method that allows the entrapment of BMP-2 over a long period of time (Figure 5A). LbL films are made of poly(L-lysine) (PLL) and Hyaluronic Acid (HA), which can be stabilized by covalent crosslinking with 1-ethyl-3-(-dimethylaminopropyl) carbodiimide (EDC). The films are post-loaded with BMP-2 by simple diffusion and retain the growth factor for at least 9 days (Crouzier et al., 2009). The amount of retained BMP-2 can be tuned by varying film thickness and the initial concentration of BMP-2 in solution. For instance, a maximal value of $1.42 \pm$ $0.26 \,\mu\text{g/cm}^2$ can be trapped in 1.4 μm thick (PLL/HA) films when the initial concentration of BMP-2 in solution is 20 µg/mL. More recently, it was shown that the crosslinking extent of the film allows the control of the amount of BMP-2 remaining in the film after a burst release (Guillot et al., 2013). This burst release depends on the crosslinking extent (7-11% for the highly cross-linked film in comparison to 62-77% for the low crosslinked films). The final amount of BMP-2 retained in the film varied (between 4 and 14 μ g/cm²) when the initial concentration was 100 µg/mL. A BMP-2 adsorbed amount of 800 ng/cm² was sufficient to trigger SMAD phosphorylation after 4 hours and ALP activity at 5 days in C2C12 cells (Crouzier et al., 2011b). In addition, BMP-2 loaded on soft films induced adhesion and

spreading, in contrast to BMP-2 added in solution. Cells also formed focal adhesions in response to matrix-bound BMP-2, suggesting a possible crosstalk between BMP receptors and adhesion receptors (e.g. integrins) (Crouzier et al., 2011b). It should be noted that for this type of films a direct comparison of the surface concentration of BMP-2 and soluble concentrations is difficult due to the difference in dimensionality (matrix-bound *versus* soluble) and molecular diffusion.

The use of temperature-sensitive polymers is another manner to electrostatically entrap BMP-2 which is already applied *in vivo* (Saito et al., 2001). The polymers can be formulated in aqueous buffers at a low temperature but become insoluble when delivered to the physiological milieu. A library of temperature-sensitive polymers has been created (Uludag et al., 2001), however only a few of them were able to retain BMP-2 for more than 5 days after the *in vivo* injection.

Entrapment by LbL techniques may be easily adapted for *in vivo* applications and some promising results have already been obtained. Indeed, hydrolytically degradable LbL coating of implants (Shah et al., 2011) was used to entrap both BMP-2 and VEGF and induced *de novo* bone formation in 4-9 weeks. Interestingly, such surface coatings can be dried and sterilized, all the while preserving BMP-2 bioactivity (Guillot et al., 2013). Clinical applications of physical entrapment-based materials can be expected in the near future.

Since the physical entrapment-based techniques are quite versatile and do not require expensive equipment, they could represent an alternative surface material to study the temporal dependence of BMP-mediated signaling. In addition, the parameters of the microenvironment, such as stiffness or growth factors presentation, can be tuned in order to analyze their effects on the BMP-2 pathway. However the nature of adhesive interactions between cells and LbL films should be clarified in order to be able to distinguish between the mere contribution of BMP-2 to signaling from the possible contribution of adhesive receptors (e.g. integrins, HA receptors), which may induce secondary signaling pathways.



Figure 5: Examples of material surfaces applied for the control BMP-2 effect on cells. Top: schematic representation of the material design. Down: cellular response to the substrates. A, Electrostatic entrapment on BMP-2 on polyelectrolyte multilayer films. C2C12 cells were plated on LbL soft films containing BMP-2 and stained for actin (red) and nucleus (DAPI, in blue). Figure adapted from (Crouzier et al., 2011b). B, Immobilization of b-BMP2 on Streptavidin gradient. Immunofluorescence images showing the nuclear translocation of the osteogenic marker Osterix, in cells grown on the BMP-2 modified surfaces. Image adapted from (Lagunas et al., 2013). C, BMP-2 immobilized to gold nanoparticle arrays produced by block copolymer micellar nanolithography. The histogram shows a comparison of SMAD1/5/8 phosphorylation levels and kinetics in cells stimulated with 1 ng of BMP-2 either added to the culture media or bound to the nanoparticles. Image adapted from (Schwab et al., 2015). D, Heparin binding peptides immobilized on a SAM captures endogenous heparin and BMP-2 from serum. The histogram shows that hMSCs area significantly increases in cells adhering to the functionalized surfaces. Image adapted from (Hudalla et al., 2011).

ii. Chemical binding of BMP-2

Biotin-Streptavidin (SAv) is the strongest non-covalent bond, which can be used to immobilize a protein on a surface following its biotinylation. This method provides not only a stable binding but also a versatile platform on which it is possible to immobilize different biotinylated compounds (Migliorini et al., 2014). The drawback consists in the need of two grafting steps, i.e. fist biotin moieties on the surface and then SAv, before growth factor immobilization. Amino-biotinylated BMP-2 added to culture media exhibits an increase in bioactivity, in contrast to carboxyl-biotinylated BMP-2 (Uludag et al., 1999). BMP-2 was amino-biotinylated and grafted on a poly(methyl methacrylate) (PMMA) thin film presenting a gradient of SAv in the range of 1.4 to 2.3 pmol/cm² (Lagunas et al., 2013) (Figure 5B). While the SAv concentration was measured by surface plasmon resonance, the binding of a BMP-2 dimer to a single SAv could be only estimated, based on the comparable size of the two proteins. However, this assumption does not consider variations in protein solubility due to aggregate formation, and the presence of non-bound biotin molecules which could change

the 1:1 ratio between BMP-2 and SAv. With such an approach, a dose-dependent osteogenic response was measured on the same substrate over a period of 6 days. Neutravidin was used to immobilize biotinylated BMP-2 (b-BMP2) on biotinylated fibronectin (b-FN) (Hauff et al., 2015) for studies on SMAD-dependent signaling and cell migration. By means of Quartz Crystal Microbalance with dissipation monitoring (QCM-D) and ELISA assays, and the amount of immobilized b-BMP-2 was detected on the surface for at least 6 days. While biotinylation is a relatively straightforward method to link proteins, it is however not site-specific and might negatively affect the biological activity of b-BMP2, when biotins are close to the BMPR binding sites. Moreover the immobilization through the small biotin moiety (~1 nm) might constrain BMP-2 spatial conformation, further inhibiting its recognition by cellular receptors.

For decades the use of covalently immobilized growth factors has been a matter of debate because of its negative impact on receptor binding and complex formation, as well as on the internalization of the protein, as discussed in section 6.2.ii. To achieve covalent binding of growth factors to supporting materials, several approaches have been developed and the use of bifunctional linkers, which target either the amino- or the carboxy-groups of the protein, is the most commonly used. Such linkers are either pre-coupled to the growth factor and then immobilized on the surface, or are at first immobilized onto the surface and then the growth factor is immobilized in a second step (Luginbuehl et al., 2004; Masters, 2011). While the former has the advantage of involving fewer preparation steps, the latter appears to be advantageous to avoid protein denaturation due to unspecific interactions with the material surface (Gonçalves et al., 2010). Additionally, the use of molecular linkers, which confer a certain degree of flexibility to the tethered growth factor, may have an impact on the mobility and accessibility of the protein for receptor binding, without loss due to diffusion. BMP-2 has been immobilized covalently to gold surfaces via a self-assembled monolayer (SAM) consisting of 11-mercaptoundecanoyl N-hydroxysuccinimide ester which binds to the free amine residues of the protein and remains bioactive for a period of 6 days without being internalized (Pohl et al., 2012).

Besides the difficulties in performing and controlling the different steps for the covalent immobilization, as well as in tailoring the immobilization strategies to the specific growth factor, a remaining challenge is to control the exact number of immobilized molecules. Thus, alternative approaches such as protein modification by expression of artificial domains or peptide tags, e.g. his-tags, have been also developed (Kashiwagi et al., 2009). So far, the biological activity of BMP-2 is often affected by such a modification in comparison with the native protein.

6.3.b. Surface patterning for the spatial control of BMP-2 presentation

To achieve control over the distribution and amount of proteins presented on materials, various strategies for surface patterning at different length scales have been developed over the last two decades. A few examples showing the patterning of BMP-2 from sub-millimeter down to nanoscale are described in the following paragraphs. These approaches may help in the future in improving the design of biomaterials as well as in deciphering BMP-2 signaling pathways (Figure 4C).

i. Sub-millimeter patterning of BMP-2

During morphogenesis, an essential long range BMP-2 gradient is formed along the ventral to dorsal axis (Ramel and Hill, 2013). *In vitro* mimicry of long-range gradients or spatially organized tissues may help deciphering the pathways of BMP-2 signaling underlying tissue formation and spatial organization. By taking advantage of the natural affinity of FN for BMP-2 (see part 6.3.c), Miller *et al.* created millimeter-sized BMP-2 patterns by printing the growth factor as a "bioink" on fibrin (Miller et al., 2009). This technique is versatile as it is possible to form patterns of various sizes and shapes, as well as gradients 1.5 mm long with different amounts of BMP-2 (from ~0.02 to ~2.245 μ g/cm²) that are deposited by overprinting BMP-2 at the same location. These BMP-2 patterns were shown to be bioactive, as assessed by ALP expression in two different cell types, namely C2C12 myoblasts and mesenchymal fibroblasts.

Another strategy consists in using microfluidics in combination with LbL technology to create millimeter-sized gradients of matrix-bound BMP-2 (Almodóvar et al., 2014). To this end, a microfluidic chamber was set in contact with a PLL/HA film and a BMP-2 gradient in solution was generated *via* passive flow pumping. As the amount of BMP-2 adsorbed onto

the film directly depends on the BMP-2 concentration in solution in the channel (Crouzier et al., 2009), a 40 mm-long gradient of matrix-bound BMP-2, ranging from 0.04 μ g/cm² to 2 μ g/cm², was thus generated. BMP-2 remained bioactive after 3 days as assessed by ALP activity in C2C12 myoblasts. This matrix-bound BMP-2 enabled the generation of a spatially controlled osteogenic differentiation, confined to the patterned area and dependent on the amount of BMP-2. Such patterns may be further used to create microtissues for studies on the effects of specific gene mutations or drugs on the formation and maintenance of bone tissues.

ii. Micrometer-sized patterns of BMP-2 on surfaces

BMP-2 patterned at the micron scale allows studies on single-cell responses. To this end, by using microcontact printing with a poly(dimethylsiloxane) stamp, Hauff et al. created 25 µmwide patterned stripes of FN onto which BMP-2 was then immobilized by biotin-neutravidin binding (Hauff et al., 2015). These patterns are stable for at least one day and b-BMP2 is not released from the stripes. Because of the discrete localization of BMP-2 molecules on the stripes, the amount of the immobilized protein on the surface is relatively high $(0.52 \text{ }\mu\text{g/cm}^2)$. The grafted BMP-2 triggers SMAD1/5/8 phosphorylation and inhibits myotube formation in C2C12 cells. Interestingly, in comparison with samples where BMP-2 was added to the culture medium, SMAD phosphorylation is prolonged over a period of 90 min, leading to a sustained localization of the SMAD complex in the nucleus. In this regard, it remains to be elucidated whether the prolonged SMAD-signaling might impact other BMP-mediated pathways. These patterned stripes served also as platform to study directed cell migration: while the migration velocity seems independent of the immobilization of BMP-2 on the patterned stripes, cells do not show any preference for a direction on the immobilized BMP-2. These results suggest it is not the binding of BMP-2 to the extracellular matrix, but rather the presentation of the proteins in gradients that might be therefore necessary to guide migration. So far, continuous surface chemical gradients of BMP-2 have been applied to study the effects of different amounts of surface-immobilized BMP-2 on cell differentiation (Lagunas et al., 2013). However, such gradients might not be steep enough to induce migratory responses. This still leaves the challenge of creating surfaces that could serve as platforms to decipher the haptotactic function of BMP-2 gradients and to study possible differences with chemotactic gradients in BMP-induced migration signaling.

To uncouple total surface density from localized density of BMP-2, microcontact printing or dip-pen nanolithography were used to produce circular micropatterned islands of BMP-2 having a diameter of 4-5 μ m (Oberhansl et al., 2014). The latter technique is based on the use of an AFM tip to deposit molecules on the surface as an ink droplet while varying the spacing between the islands. For the chemical binding of BMP-2 to the surface, either a thiolated biotin linker or a thiolated biotin lipid layer was first placed on gold-coated substrates using the micropatterning approaches. Following incubation with SAv, b-BMP2 was immobilized on the patterned regions and remained bioactive. Cell differentiation was comparable to non-patterned BMP-2 on the surface, when taking into account the estimated total surface density of the protein. When considering the impact of the local density of BMP-2 on cell response, these studies suggest that BMPR oligomerization might be favored when the growth factor is presented in discrete regions, thus leading to more efficient signaling, but this remains to be elucidated.

iii. Nanoscale surface patterning of BMP-2

Materials which allow the control of cell responses at the nanoscale are of special interest, being at the length scale of BMP-2 and BMPRI and II interactions. Nanoscale modifications of surfaces carrying BMP-2 have been applied to study the influence of substrate modifications on osteogenic differentiation by changing their physicochemical properties (Kim et al., 2013), or for determining the effect of the surface density of BMP-2 on cell signaling (Schwab et al., 2015). In the first case, nanogrooves and nanodots ranging between 150-300 nm and 460 nm in size, respectively, consisting of polyurethane acrylate and coated with poly(glycidyl methacrylate), were functionalized with BMP-2 peptides. The presence of nanoscale features on the surface improves calcium deposition and the expression of osteogenic markers, which are even enhanced in presence of BMP-2 peptides. Better tuning of the nanostructure size to allow the formation of focal adhesions (Cavalcanti-Adam et al., 2006) and quantifying the amount of BMP-2 peptides immobilized on the surface may help to further use these new nanotopography tools to study BMP-mediated signaling
To achieve control over BMP-2 surface density, gold nanostructured substrates produced by block copolymer micellar nanolithography were recently applied as a substrate for the immobilization of BMP-2 using a bifunctional linker, as described in 6.3.ii, now coupled to gold nanoparticles (Figure 5C) (Schwab et al., 2015). The coupling of BMP-2 heterodimers to every single nanoparticle on the surface was detected and quantified at the single molecule level by AFM, thus making it possible to experimentally determine the amount of immobilized growth factor on the surface. Additionally, with this nanopatterning technique it is possible to vary the amount of immobilized BMP-2 by varying the interparticle spacing and to achieve the controlled immobilization of amounts which are below the lowest value reported previously (31 ng/cm²) (Karageorgiou et al., 2004). Interestingly, the bioactivity of the immobilized protein shows a characteristic regulation of SMAD phosphorylation levels and kinetics, which differs from those triggered by BMP-2 added to the cell culture medium. In fact, when BMP-2 is immobilized on the surface, regardless of the amount used (ranging from 0.2 to 3.3 ng/cm²), SMAD phosphorylation onset is delayed but then is still maintained over a long period of time (180 min). Additionally, while the lowest amount of BMP-2 added to the culture media is not sufficient to activate the SMAD complex, the corresponding concentration immobilized on the surface leads to a remarkable SMAD phosphorylation. This study indicates that the sustained presentation rather than the amount of BMP-2 regulates SMAD-signaling, suggesting a different temporal regulation of BMP-mediated signaling pathways when the growth factor cannot be internalized. One hypothesis is that the immobilization might affect lateral receptor mobility and oligomerization on the one hand. On the other hand, when the receptors cannot be internalized in a complex with the ligands, the number activated receptors and their internalization rates might be different than those in presence of BMP-2 in the media.

6.3.c. Materials inspired by the interaction of BMP-2 with ECM components

One of the ECM functions is to serve as a reservoir of growth factors via a large variety of interactions (for example electrostastic, hydrogen-bonds, hydrophobic, Van der Waals). This type of interaction is important for growth factor release in soluble phase, orientation and therefore signaling. ECM presents epitopes which bind growth factors to limit their diffusion and maintain their activity locally. Therefore, the incorporation of BMP-2 binding sites of

the ECM on materials would permit BMP-2 sequestration in a non-covalent manner (Hudalla and Murphy, 2011) (Figure 4D).

i. Modulation of the activity of BMP-2 bound to glycosaminoglycans

Glycosaminogycans (GAG) are major polysaccharide components of the ECM. These biopolymers can be divided into four groups: HA, the only not sulfated, heparin sulfate (HS), chondroitin sulfate (CS) and dermatan sulfate (DS). GAGs bind growth factors with a low binding constant (Maciag et al., 1984; Nugent and Edelman, 1992), mainly due to electrostatic interactions. It has been shown that BMP-2-GAG binding could either up- or downregulate BMP-2 cellular activity (Bramono et al., 2012; Jiao et al., 2007; Kuo et al., 2010). Ruppert *et al.* (Ruppert et al., 1996) demonstrated that the BMP-2 homodimer has an heparin-binding site at its N-terminus. The binding seems to be due to the interactions between the basic residues of the Hp-binding site and the sulfate groups presented on GAGs. In particular, it has been demonstrated by surface plasmon resonance that GAGs alter the binding between BMP-2 and its receptor IA in a sulfation-dependent manner (Hintze et al., 2014).

Hp can be used as a material coating to present BMP-2 to cells. For example titanium substrates modified with Hp to present BMP-2 promote osteoblast function, osteointegration, and bone regeneration *in vitro* and *in vivo* (Kim et al., 2011, 2014). Resorbable polymer (poly(L-lactic acid) and poly(β -caprolactone) films, covalently functionalized with oriented Hp, linked *via* reductive amination, immobilize BMP-2 and improve cell attachment and proliferation (Edlund et al., 2008). However, the surface functionalization with Hp is only qualitative: with these materials it is not possible to achieve a precise quantification of both GAG and growth factor and to characterize the BMP-2 release during cell culture. Moreover, changes in mechanical properties after Hp coating might also influence cell behavior by changing cell-substrate forces and activating cytoskeleton rearrangements.

A different way to exploit the use of surfaces functionalized with Hp to bind growth factors has been proposed by Hudalla *et al.* (Hudalla et al., 2011) (Figure 5D). Here a SAM presenting Hp-binding peptides and RGD peptides was used to specifically bind the

endogenous Hp, which is complexed with the growth factors present in the cell medium. Thanks to the inert SAM background, these surfaces avoid the non-specific binding of other components of the serum and reduce the need of high non-physiological concentrations of growth factors. Human MSCs plated on SAM substrates show an enhancement of the BMP signaling pathway, and therefore an enhanced cellular proliferation and osteogenic differentiation. Hp and HS have been extensively used in 3D scaffolds due to their synergistic effect on BMP-2 activity. Other reviews extensively report the use of Hp for drug delivery and for *in vivo* applications (Hudalla and Murphy, 2011; Sakiyama-Elbert, 2014). Even though promising for clinical applications, these approaches do not provide structural and stoichiometric information of the GAG/BMP-2 binding. The combined effects of GAGs on BMP-2 cellular responses could lead to the hypothesis that HS proteoglycans function as correceptors for BMP-2. Thus, it is crucial to get clear information on the GAGs structural and conformational modifications after BMP-2 binding. Functionalized surfaces together with surface-sensitive techniques could provide useful tools for answering this question.

A detailed surface-based study was proposed by the Svedhem group (Altgärde et al., 2013) using CS, an important structural ECM component. CS, covalently attached to supported lipid bilayers, binds BMP-2 and cells spread in response to BMP-2. Although the bioactivity of BMP-2 in these conditions was not verified, this type of model assembly opens new possibilities for the study of BMP-2 interactions with biopolymers in controlled environments.

HA is a GAG which possesses highly interesting physical and mechanical properties. By interacting with water molecules, HA provides the tissue with the ability to resist compression stresses (Haxaire et al., 2003). HA alone has a positive effect on cell proliferation and upregulates osteogenic markers (Zou et al., 2004). Several studies have described that BMP-2 can be trapped in HA crosslinked gels (Bhakta et al., 2012; Kim and Valentini, 2002; Kisiel et al., 2012; Martínez-Sanz et al., 2011), and its retention can be improved using different strategies. For instance, Kisiel *et al.* (Kisiel et al., 2013) precomplexed BMP-2 with DS or with Hp to increase its affinity for HA. Thus it is possible to load three times more pre-complexed BMP-2 in HA hydrogels than free BMP-2. The retention of the pre-complexed BMP-2 is significantly higher than free-BMP-2 on HA gels after 30 days. Alternatively, HA can be chemically modified to better retain BMP-2. For instance, bisphosphonates can be grafted onto HA, which leads to a 8-fold increase in the retention capacity for BMP-2 in comparison with pure HA gels (Hulsart-Billström et al.,

2013). HA can also be associated in LbL with positively-charged polypeptides to create thin self-assembled films that can be deposited on material surfaces, as described in 6.3.i (Crouzier et al., 2009). This presentation mode maintains the biological activity of BMP-2, as confirmed by SMAD phosphorylation. Interestingly, the phosphorylation signal is increased in cells cultured on the matrix-bound BMP-2 soft films in comparison with the stiff ones (Crouzier et al., 2011b). Indeed, these biomimetic substrates combine both physical and chemical cues, thereby opening new possibilities to investigate the importance of BMP-2 in mechanotransduction. In fact, BMP signaling appears to be closely connected to mechanotransduction pathways at several levels. During embryonic development, for example, both BMP-2 gradients and mechanical signals such as tissue stiffness and compressive forces contribute to tissue polarity and patterning (Kopf et al., 2014), although a deep understanding of the exact mechanisms is still missing.

ii. Co-presentation of BMP-2 and cell binding motifs

Many efforts have been taken to engineer the environment so that it is supportive of both adhesion and differentiation in a controlled manner. However, the presentation of multiple and defined cues at the cell-material interface is still a challenge and so far the main focus has been on the effects on long-term responses and *in vivo* applications, whereas information on the signaling pathways and crosstalk is still missing. An emerging approach is the copresentation of integrin-binding motifs and BMP-2 (Figure 4D). Here, adhesion peptides such as RGD or collagen peptides are immobilized on the material surface to induce integrinmediated adhesion (García and Reyes, 2005; Lee et al., 2011; Martino et al., 2014; Shekaran et al., 2014).

Several studies from the Hubbell group have demonstrated that BMP-2 binds to ECM proteins like FN (Martino and Hubbell, 2010), tenascin C (De Laporte et al., 2013), fibrinogen, but not to collagen I (Martino et al., 2013, 2014). In particular, FNIII₁₂₋₁₄ binds BMP-2 and other growth factors in a promiscuous manner, with a K_D in the nanomolar range and without affecting the biological activity of the growth factors (Martino and Hubbell, 2010). Engineered substrates made with fibrin molecule carrying a peptide containing FNIII₁₂₋₁₄ permit a greater retention of the factors with respect to normal fibrin matrices. Fibrin matrix itself and its heparin-binding domain could promiscuously bind several growth

factors, including BMP-2 (Martino et al., 2013). Fibrin-synthetic matrices presenting both the fibrin heparin-binding domain inside a polymeric scaffold and growth factors, like fibroblast growth factor-2 (FGF-2) and Platelet-derived growth factor-2 (PDGF-2), have been successfully tested *in vivo*. The proximity between the RGD motif present in FNIII₉₋₁₀ and the growth factor binding site on FNIII₁₂₋₁₄ serves as rationale for the use of such peptides to allow synergy with BMP-2 and potentiate bone formation (Martino and Hubbell, 2010). The synergistic interaction between immobilized collagen I and BMP-2 in osteogenic differentiation of MSCs has recently been investigated using a microcontact printing platform (Rasi Ghaemi et al., 2016).

To achieve more defined responses, the immobilization of adhesive motifs and BMP-derived peptides on material surfaces have been also performed. The immobilization of BMPpeptides has been applied to various materials, including polymers and hydrogels, but here we will focus on two examples where the molecules have been grafted onto 2D surfaces. Zouani et al., (Zouani et al., 2010) grafted RGD and BMP-2 mimetic peptides on polyethylene terephthalate to enhance osteogenic differentiation. The impact on osteogenic differentiation of the co-presentation of RGD and BMP-bioactive peptides carrying an azide group has been also investigated at concentration gradients on self-assembled monolayers generated by UVO treatment (Moore et al., 2011). Osteopontin and BMP-2-derived motifs have been also immobilized by engineering a cysteine residue and 12-aminoacid stretch switch tag to address the C-terminus of the peptides (Mitchell et al., 2010). These strategies rely on the use of BMP-derived peptides based on the sequence of the knuckle epitope of a BMP-2 monomer comprising the low affinity site for binding to BMPRII (Nickel et al., 2001). However, this is in contrast with biochemical studies showing that two knuckle epitopes should be present on one BMP-2 molecule in order to achieve receptor activation, since depletion of a single epitope results in complete loss of ALP activation (Knaus and Sebald, 2001). This leaves the question whether the surface immobilization strategies might unveil otherwise masked activities of the BMP-2 molecule which are not possible to investigate with BMP-2 in solution.

6.4. Concluding remarks and perspectives

Recently new aspects in BMP-mediated signaling have been unraveled, pointing out the need to design and develop new approaches for BMP-2 delivery. In view of future clinical applications, some critical questions regarding BMP-2 presentation and functions remain to be solved in order to provide innovative solutions for bone tissue engineering. It is therefore important to engineer materials that can present BMP-2 in a spatially and temporally controlled manner.

In this review, we have shown that several technical solutions have now been developed to present BMP-2 in a controlled manner to cells, using either covalent grafting, physical entrapment or interactions with ECM components, which precisely tune the activity of BMP-2 and control its orientation. The use of two-dimensional surfaces offers the advantage of being controllable with surface-sensitive techniques and compatible with high resolution microscopy. Some of the technical approaches here described, such as physical entrapment of BMP-2 and GAG-based materials, might be soon applied to scaffolds for tissue engineering applications. In vitro studies with BMP-2-presenting surfaces could allow the deciphering of hidden biological functions of BMP-2. For instance, materials on which BMP-2 and ECM ligands (adhesion ligand and/or GAGs) are co-presented in a spatially controlled manner could provide important information on the crosstalk between adhesion (e.g. integrins) and BMP-2 signaling pathways. Super-resolution microscopy techniques could be helpful to clarify the interactions at the cell membrane between BMP-2 and its receptors, explaining the dynamics of receptor recruitment and mobility, as well as the architecture of receptor complexes. By combining the spatio-temporal control over BMP-2 presentation on surfaces and high-resolution imaging techniques it should also be possible to elucidate the regulation of BMP-2 receptor endocytosis and its impact on signaling pathways. Certainly there is a need to develop labeling strategies to track BMP-2, as well as BMPRs, without affecting their biological activity and signaling kinetics. Recent attempts have shown that BMP-2 activity is significantly slower when fluorophores are coupled to the growth factors (Alborzinia et al., 2013). Thus, the development of new biochemical tools becomes essential: for example, the conjugation of BMP-2 to various types of linkers should be in a site-specific manner, to permit the control of its orientation, once grafted on surfaces, and to improve the bioactivity of covalently-grafted BMP-2. Moreover, biochemical and structural studies at the

molecular level could also help in improving our knowledge of the mechanisms of BMP-2 binding to GAGs and to ECM proteins, which is largely incomplete at present.

In conclusion, innovative solutions in bone regenerative medicine are needed to repair critical bone defects. Surface materials with controlled delivery and presentation of BMP-2 can be used to direct cell signaling for bone repair. In the future, through a joint effort from material and biological sciences, it should be possible to further improve the presentation of BMP-2 at the cell surface. The knowledge gained from *in vitro* studies, using well-defined materials platforms, may open new ways for regenerative therapies.

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Objectifs de la thèse et stratégies

Nous avons vu que les systèmes adhésifs caractérisent les tissus et peuvent affecter la dynamique de leur formation. Le répertoire d'intégrines et de cadhérines est en effet propre à un type cellulaire et à son niveau de différenciation. Cette adaptation du répertoire du système adhésif permet de répondre à différentes fonctions. Dans les étapes précoces de la formation tissulaire à partir de cellules isolées, la migration et la multiplication cellulaires sont cruciales, et par elles le rôle des récepteurs d'adhésion cellules/matrice. Plus tardivement dans cette différenciation et après prolifération des cellules, les contacts cellulaires se forment, ce qui met en jeu les récepteurs d'adhésion cadhérines.

Pour comprendre le rôle de ce système adhésif dans la différenciation cellulaire, il est courant de stimuler une intégrine spécifique, par une protéine de la matrice ou un peptide particulier, et de regarder les effets sur les facteurs de transcription et l'adaptation du répertoire de cadhérines. Cette stratégie, consistant à stimuler un récepteur d'adhésion particulier, permet de voir l'influence des protéines adhésives de la matrice extracellulaire sur l'adhésion initiale et la différenciation cellulaire. Cependant, elles sont inadaptées pour comprendre comment les cellules se différencient et forment un tissu *de novo*, sans environnement matriciel prédéfini mais au contraire en créant leur propre microenvironnement. Est-ce la sécrétion de protéines matricielles qui engendre un changement du répertoire des récepteurs d'adhésion, ou est-ce le changement d'intégrines qui permet la sécrétion et le remodelage des protéines de la matrice ?

Dans le cas d'un défaut osseux, nous avons vu qu'il y avait de nombreux échanges biochimiques et mécaniques entre le muscle et l'os. De plus, bien qu'aucune preuve n'ait été apportée sur leur réelle contribution *in vivo*, les myoblastes pourraient contribuer à réparer le tissu osseux. La transdifférenciation de myoblastes vers des cellules osseuses peut notamment être guidée par la BMP-2 qui est un facteur de croissance ostéoinducteur produit par les cellules osseuses. En conditions normales, les myoblastes étant destinées à se

différencier en muscle, elles n'expriment aucun marqueur de différenciation osseuse. L'utilisation de ces cellules permet donc d'étudier l'effet spécifique de la BMP-2 comme unique chef d'orchestre dans la différenciation osseuse.

Notre stratégie est d'utiliser la BMP-2 comme premier stimulus pour guider les cellules vers la différenciation osseuse. Nous regardons tout d'abord le rôle des récepteurs d'adhésion (intégrines) à temps initial et leurs implications dans un jeu croisé avec les récepteurs BMP. Puis nous regardons l'expression au cours du temps des récepteurs d'adhésion intégrines et cadhérines lors de la formation du tissu, lorsque la cellule s'adapte à son nouvel environnement, en association à sa différenciation.

Les biomatériaux que nous utilisons sont les films de polyélectrolytes à base de (PLL/HA), pour lesquels nous avons déjà montré que la rigidité et le mode de présentation de la BMP-2 pouvaient être découplés (Figure 22). En effet, sans BMP-2, les cellules n'adhèraient que les substrats rigides (verre et STIFF films). Les cellules sont donc sensibles à la rigidité. La présence de BMP-2 soluble ne change pas la réponse cellulaire en termes d'adhésion et d'étalement. En revanche, lorsque la BMP-2 est liée à la matrice, les cellules adhèrent et s'étalent même sur films mous. Ceci met en évidence la sensibilité de la cellule au mode de présentation de la BMP-2. Dans ce travail préliminaire, il avait été observé que les cellules forment des plaques focales, ce qui suggère que des récepteurs d'adhésion intégrines sont impliqués dans la réponse cellulaire à la BMP2.



Figure 22 : Effet de la rigidité et du mode de présentation de la BMP-2 sur l'adhésion et l'étalement des C2C12. Les cellules sont observées après 16h d'adhésion. Sur substrat rigide (verre et STIFF films), les cellules s'étalent dans toutes les conditions, tandis que sur substrat mou (SOFT film) seule une BMP-2 liée à la matrice permet aux cellules d'adhérer et de s'étaler. (Crouzier et al., 2011b)

Suite à ces travaux préliminaires, nous nous sommes intéressés, dans ma thèse, à répondre aux questions suivantes (Figure 23) :

1- Les intégrines sont-elles impliquées dans la réponse cellulaire à la protéine BMP-2 présentée par le biomatériau ?

Pour répondre à cette question, la signalisation associée aux récepteurs d'adhésion et celle au BMP2 ont été étudiées (Fourel, Valat et al, 2016).

2- Quel est l'équilibre entre les intégrines (cellule/matrice) et les cadhérines (cellule/cellule) au cours de la lors de la transdifférenciation de myoblastes en cellules osseuses induite par la BMP2 ?

Pour ce faire, des facteurs de transcription caractéristiques des étapes de la formation du muscle et de l'os ont été suivis et la formation d'une nouvelle matrice extracellulaire a aussi été caractérisée au niveau de l'expression génique et protéique (Valat et al, en préparation).



Figure 23 : Représentation schématique des objectifs de la thèse

Chapitre II.

Coopération entre l'intégrine β3 et les récepteurs BMP dans l'étalement cellulaire et la réponse SMAD

Cette première partie fait l'objet d'un article publié dans *Journal of Cell Biology* 2016 Mar 14; 212(6):693-706 Doi: <u>10.1083/jcb.201508018</u>

1. Résumé

1.1. Introduction

De nombreuses études rapportent une synergie entre les voies de signalisation des récepteurs d'adhésion cellule/matrice (les intégrines) et celles des facteurs de croissance (Comoglio et al., 2003; Ivaska and Heino, 2011; Margadant and Sonnenberg, 2010) sans toutefois expliquer clairement les coopérations entre les BMP-récepteurs (BMPRs) et les intégrines. Nous ne savons toujours pas quel récepteur initie la signalisation et si un tel jeu croisé implique i) une interaction à la membrane ou ii) une coopération entre les voies de signalisation en aval. La difficulté vient des conditions expérimentales qui ne permettent pas de discriminer la présentation du facteur de croissance des propriétés physiques de la MEC. En effet, les études *in vitro* sont généralement faites sur plastique, dont la rigidité est largement supérieure à ce qui est mesuré *in vivo*, avec de la BMP-2 en solution et non enchâssée dans la matrice.

Récemment, nous avons montré que les films de poly-électrolytes (PLL/HA) pouvaient être utilisés pour présenter la BMP-2 de manière liée à la matrice pour contrôler la différenciation cellulaire en induisant une différenciation osseuse *in vitro* et *in vivo* (Crouzier et al., 2009, 2011a). Nous avons aussi montré que la BMP-2 liée à la matrice affectait l'étalement et la migration cellulaire (Crouzier et al., 2011b).

Dans cette étude, notre but est de comprendre comment la signalisation des intégrines et celle de la BMP-2 sont biochimiquement interprétées par la cellule et connectées par la signalisation SMAD. Pour aller plus loin dans cette étude, nous avons découplé les signaux mécaniques de la MEC et le signal biochimique de la BMP-2 en utilisant les films (PLL/HA). Nous avons étudié comment les signaux biochimiques fournis par la BMP-2 liée à la matrice peuvent affecter la réponse mécanique et la différenciation cellulaire.

1.1. Résultats

Nos résultats ont montré que la BMP-2 présentée par la matrice, à travers son interaction avec les BMPRs, initie l'adhésion cellulaire indépendamment de la rigidité du substrat. En effet, des mesures d'étalement après déplétion des BMPR ont mis en évidence l'implication du BMPR-Ia, et dans une moindre mesure du BMPR-II, dans l'adhésion et l'étalement des C2C12 sur des substrats mous chargés en BMP-2. Nous avons cherché à savoir si un récepteur d'adhésion cellule/matrice (intégrine) serait lié à cette réponse mécanique. A temps initial, les myoblastes C2C12 expriment majoritairement les intégrines β 1 et β 3. Pour cette étude de la réponse à la BMP-2 à temps court, nous nous sommes donc focalisé sur ces deux récepteurs. L'inhibition de l'intégrine β 3, et non β 1, entraîne une diminution de l'adhésion et de l'étalement cellulaire. Ainsi, l'étalement des myoblastes sur films mous chargés en BMP-2 résulterait d'une coopération, directe ou indirecte, entre les récepteurs BMP et l'intégrine β 3. De plus, la BMP-2 chargée dans les films induit une augmentation de la migration cellulaire. Nos résultats ont montré que cela est dû à une augmentation de la dynamique des sites d'adhésion.

Réciproquement, la déplétion de l'intégrine β 3, et non de l'intégrine β 1, induit une baisse de l'activation de la voie Smad observée par une diminution de la phosphorylation de SMAD1,5,8 en Cter (suivie en Western Blot à 4 h) et de son activité transcriptionnelle (mesurée à 15 h par l'utilisation de C2C12 transfectées avec le plasmide BMP responsive element-luciferase). L'intégrine β 3 joue donc un rôle dans la réponse SMAD à la BMP-2.

Finalement, nous proposons un modèle dans lequel l'intégrine β 3 agirait à différentes étapes de la réponse SMAD induite par la BMP-2 en contrôlant à la fois la phosphorylation de SMAD par les BMPRs et la stabilité de pSmad1^{Cter} par la répression de l'activité de GSK3 β .

2. Article

β3 integrin-mediated spreading induced by matrix-bound BMP-2 controls Smad signaling in a stiffness-independent manner

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ABSTRACT:

Understanding how cells integrate multiple signaling pathways to achieve specific cell differentiation is a challenging question in cell biology. We have explored the physiological presentation of BMP-2 by using a biomaterial which harbors tunable mechanical properties to promote localized BMP-2 signaling. We show that matrix-bound BMP-2 is sufficient to induce β 3 integrin-dependent C2C12 cell spreading by overriding the soft signal of the biomaterial and by impacting actin organization and adhesion site dynamics. In turn, $\alpha\nu\beta$ 3 integrin is required to mediate BMP-2-induced Smad signaling through a Cdc42/Src/FAK/ILK pathway. β 3 integrin regulates a multi-step process to control first BMPR activity and second the inhibitory role of GSK3 on Smad signaling. Overall, our results show that BMP receptors and β 3 integrin work together to control Smad signaling and tensional homeostasis, thereby coupling cell adhesion and fate commitment - two fundamental aspects of developmental biology and regenerative medicine.

2.1. Introduction

Mechanotransduction enables cells to sense and adapt to forces and physical constraints imposed by the extracellular matrix (ECM) (Schwartz, 2010; Vogel and Sheetz, 2006). ECM supports morphogenetic processes during embryonic development or cancer and during tissue homeostasis in adulthood. Apart from providing a structural support, the chemical and physical properties of the ECM controls tissue architecture by driving specific cell differentiation programs (Mammoto and Ingber, 2010). Soluble growth factors are chemical cues incorporated into the ECM. Their distribution, activation, and presentation to cells are spatially regulated by the physical properties of the ECM (Discher et al., 2009; Hynes, 2009b; Tenney and Discher, 2009). However whether growth factors are able to initiate a mechanical response is still a matter of debate. Although it is known that cell mechanics control gene transcription for the maintenance of pluripotency, the determination of cell fate, pattern formation and organogenesis (Gilbert et al., 2010; Lu et al., 2012; McBeath et al., 2004), the signaling pathways regulating the activity of nuclear transcription factors in response to these physical signals are not well understood.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor- β (TGF- β) superfamily. They have been shown to participate in patterning and specification of several tissues and organs during vertebrate development. They regulate cell growth, apoptosis and differentiation in different cell types (Capdevila and Izpisua Belmonte, 2001; Massague, 2000). BMP-2, BMP-4 and BMP-7 are key molecules for normal bone development in vertebrates and induce osteoblastic differentiation of C2C12 mesenchymal pluripotent cells (Katagiri et al., 1994). Early events in BMP signaling are initiated through the phosphorylation of specific receptor-regulated Smad proteins, namely Smad1, Smad5 or Smad8. After phosphorylation, R-Smads form heteromeric complexes with the common mediator Smad4. These Smad complexes translocate to the nucleus and activate the transcription of specific target genes (Massague and Wotton, 2000). Besides its role in bone differentiation, BMP-2 appears to control cytoskeletal rearrangements and cell migration, suggesting a role in mechanotransduction (Gamell et al., 2008; Kopf et al., 2014). However, little is known about the pathways involved in BMP-2-mediated cell adhesion and migration. Several studies have reported synergistic effects between integrin mechanoreceptors and growth factor signaling pathways (Comoglio et al., 2003; Ivaska and Heino, 2011; Margadant and Sonnenberg, 2010) without a particular focus on integrins and BMP receptor cooperation. Whether these BMP responses depend on the recruitment of integrin mechanoreceptors or on the cross-talk with additional pathways remains to be elucidated. It is still not known which receptor initiates signaling and whether such cross-talk involves (i) membrane-proximal interactions or (ii) cooperation in the downstream signal transduction pathways. The difficulty comes from used experimental conditions which do not discriminate between growth factor presentation (usually diluted in culture medium) and ECM physical properties (imposed by the material on which cells are cultured).

We have shown that a biomimetic material can be used to present BMP-2 in a matrix-bound manner to control cell fate by inducing bone differentiation in vitro and in vivo (Crouzier et al., 2009, 2011b). We have also shown that matrix-bound BMP-2 affects cell spreading and cell migration (Crouzier et al., 2011b). Here, our goal was to understand how integrin and BMP-2 signaling are biochemically interpreted and connected through the BMP-2-induced Smad cascade. To gain insight into the possible cross-talk between BMP and adhesion receptors, we uncoupled ECM stiffness from biochemical signals transduced by BMP-2 using a biopolymeric biomaterial. We investigated how biochemical cues provided by matrix-bound BMP-2 may affect cell mechanical responses and drive a genetic program. We show that BMP-2 receptors and β 3 integrins cooperate and coordinate a cellular response to control both cell spreading and Smad signaling. The spatial organization of BMP-2 presented in a "soft matrix-bound" manner is sufficient to trigger cell spreading and migration overriding the stiffness response through actin and adhesion site dynamics. In turn $\alpha\nu\beta3$ integrin is required for BMP-2 induced Smad signaling by controlling both BMPR activity and Smad stability. Our data show that BMP and integrin signaling converge to couple cell migration and fate commitment.

2.2. Results

2.2.a. Matrix-bound BMP-2/BMPR interaction alters the stiffness response of C2C12 cells

To mimic *in vitro* the likely context of BMP-2 presentation *in vivo*, we used a thin biomaterial made by self-assembly of hyaluronan (HA) and poly(L-lysine) (PLL). Adapting the crosslinker concentration to obtain either low cross-linked (low CL) or high cross-linked (high CL) films enabled us to modulate film stiffness (**Table S1**) as previously described (Boudou et al., 2011; Crouzier et al., 2011b). Hereafter, low CL and high CL films will be named soft and stiff conditions, respectively. BMP-2 is simply post-loaded on the film in order to obtain matrix-bound BMP-2 (bBMP-2) as the film presents high affinity towards BMP-2 (Crouzier et al., 2009). The amounts of loaded BMP-2 were similar for soft and stiff films with 740 + 120 ng/cm^2 and $970 + 180 \text{ ng/cm}^2$ of adsorbed BMP-2 respectively. These biomimetic films offer the advantage of presenting BMP-2 to cells in a matrix-bound manner and promote localized BMP-2 signaling. They are truly unique in their ability to present BMP-2 to cells in a matrix-bound manner, as BMP-2 is a very sensitive protein able to quickly lose its bioactivity and difficult to graft on surfaces in controlled amounts (King and Krebsbach, 2012). The films behave as nano-reservoirs for stable and bioactive BMP-2 molecules (Fig. S1A). They can turn on BMP-responsive element luciferase reporter gene (BRE-Luc) (Fig. S1B), phosphorylation of BMP-2-regulated transcription factors Smad at the C-terminus (pSmad1^{Cter}) (Fig. S1C), expression of alkaline phosphatase (ALP) (Fig. S1D) and they induce bone growth in vivo at ectopic site (Crouzier et al., 2011a).

C2C12 myoblasts represent a well-accepted *in vitro* model system to study the ability of BMP-2 to alter cell lineage from the myogenic to the osteogenic phenotype (Katagiri et al., 1997; Yamamoto et al., 1997). As expected, plastic substrate (TCPS) or films of different stiffness did not activate the Smad signaling pathway in the absence of BMP-2 (**Fig. S1**). Compared to delivery of soluble BMP-2 (sBMP-2), the presentation of matrix-bound BMP-2 (bBMP-2) potentiated the Smad response in cells on soft films whereas it did not improve the Smad response in cells grown on stiff films (**Fig S1**). This reveals interference between substrate stiffness and BMP-2 signaling, stressing the necessity of working under conditions of high matrix compliance when attempting to elucidate BMP-2-mediated cell signaling. We then compared C2C12 cell spreading at early times (4h). This time point corresponds to an optimal spreading (3 fold increase between 30 min and 4h) and avoids the large variability

in the kinetics of cell spreading on the polyelectrolyte films at earlier time points (Crouzier et al., 2011b). Of note C2C12 cell spreading on low CL films with bBMP-2 can be maintained for at least 24 h (Fig. S2A). As anticipated from our previous experiments (Ren et al., 2010) and from reports in the literature of natural and synthetic gels of various stiffness (Discher et al., 2005), the cells spread more on stiff films than on soft ones (Fig. 1A and 1A'). On stiff films, exposure to matrix-bound BMP-2 did not induce any changes in cell adhesion or spreading (Fig. 1A and 1A'). In contrast, whereas C2C12 myoblasts were round and poorly spread on soft films in the absence of BMP-2 or with exposure to sBMP-2, exposure to bBMP-2 induced a drastic increase in cell adhesion and spreading (Fig. 1A and 1A'). We examined whether cell spreading was initiated by BMP-2 receptors after sensing matrixbound BMP-2. To do so, we investigated whether knockdown of BMPR-Ia and of BMPR-II, known to be expressed in C2C12 cells (Nohe et al., 2002), could impact C2C12 cell spreading induced by BMP-2 bound to soft films (Fig. 1B and 1B'). The efficiency of siRNA-mediated BMPR-Ia and BMPR-II silencing was determined by qPCR analysis, which showed a specific decrease of targeted mRNA expression (Fig. 1C). C2C12 cell spreading was strongly reduced in response to bBMP-2 on soft films after BMPR-Ia or BMPRII receptor depletion

(Fig. 1B and 1B').

Our results indicate that BMPR-Ia receptors and to a lesser extent BMPR-II receptors are involved in C2C12 myoblast spreading induced by soft matrix-bound BMP-2. The biomaterial provides BMP-2 confinement and promotes localized BMP-2 signaling which is sufficient to induce cell spreading independently of substrate stiffness. In other words, matrix-bound BMP-2 alters the stiffness response of C2C12 cells via interactions with BMPR.

Chapitre II. Coopération entre l'intégrine β 3 et les récepteurs BMP dans l'étalement cellulaire et la réponse SMAD



FIGURE 1. Soft matrix-bound BMP-2 is sufficient to induce cell spreading. (A, left) C2C12 cells morphology observations after 4 h of plating on the biopolymeric films with soluble BMP-2 (sBMP-2) or matrix-bound BMP-2 (bBMP-2). Actin and nucleus staining of C2C12 cells revealed a well spread morphology on high CL films in the absence of BMP-2 or presence of sBMP-2 or bBMP-2. In contrast, for cells on low CL films, bBMP-2 induced a striking increase of cell spreading as compared to sBMP-2. (A, right) Quantification of cell number and spreading shows the drastic increase in cell spreading in response to soft matrix-boundBMP-2. (B, left) C2C12 cells were depleted in BMPR-Ia or BMPR-II using siRNA. (B, right) After 4 h of plating, cell area on soft matrix-bound BMP-2 was quantified by visualizing cells Factin. (C) Confirmation of efficiency of BMPR-Ia and BMPR-II deletion by qPCR analysis. Scale bar: 100 µm. Data are means <u>+</u> SEM. 60 cells per condition are analyzed (*n*=3). NS, not significant;*p < 0.05, **p < 0.005

2.2.b. \$\beta3 integrin is required for cell spreading in response to matrix-bound BMP-2

As integrins play a key role in adhesion, spreading, and mechanotransduction (Albiges-Rizo et al., 2009) and in particular in early adhesion of myoblasts and their subsequent fusion to form myotubes (Mayer, 2003), we investigated their possible involvement in cell spreading

induced by BMP-2 ligand bound to the soft matrix. The appearance of focal adhesions as assessed by vinculin staining, and the development of actin stress fibers were correlated with cell spreading on matrix-bound BMP-2 to soft films (**Fig. 2A**). Moreover, manganese treatment was sufficient to induce cell spreading in the absence of BMP-2 (**Fig. 2B**) suggesting that cell spreading might be the result of integrin activation which was visualized by the increase in vinculin containing focal adhesions (**Fig. 2B**). Of note, bBMP-2 induced cell spreading was also observed in the absence of serum (**Fig. S3A** and in (Crouzier et al., 2011b), excluding the presence of a soluble mediator in the serum.

To gain further insight into the spatial organization of adhesion receptors in C2C12 cells spread on matrix-bound BMP-2 films, we labelled integrins using specific antibodies. Only very small clusters of integrins were visible at the surface of cells spread in the absence of matrix-bound BMP-2 (**Fig. 2C top**). Conversely, we observed that matrix-bound BMP-2 induced an increase in integrin receptor clustering and the organization of focal adhesions containing α 5, α V, β 1 and β 3 integrins at the basal cell surface (**Fig. 2C bottom**). To confirm the role of β 1 or β 3 integrins, we investigated whether integrin-blocking antibodies (**Fig. 2D**) or knockdown by RNA interference (**Fig. 2E and 2E'**) could affect cell spreading induced by matrix-bound BMP-2 on soft films. The effect of siRNA-mediated silencing of β 1 or β 3 chains was efficient and identical, as judged by Western blot analysis (**Fig. 2F**). Strikingly, blocking β 3 integrin greatly decreased the number of adherent and spread cells as compared to β_1 integrin blockade (**Fig. 2D**). Using integrin-blocking antibodies against α chains revealed that α_V integrins were also implicated in the process of cell spreading suggesting the involvement of $\alpha_V \beta_3$ integrins in BMP-2 mediated cell spreading (**Fig. S2B**).

Our results show that matrix-bound BMP-2 induces $\alpha\nu\beta3$ integrin clustering. We next determined whether $\beta3$ integrin is engaged with a ligand in these BMP-2-induced focal adhesions. To do so, we set up a competition assay using cyclic RGD (cRGD), a specific ligand for $\beta3$ integrin (Dechantsreiter et al., 1999), to compete with potential endogenous ligands. We showed that cRGD inhibited cell spreading onto BMP2-soft matrix in contrast to the negative control cRAD (**Fig. S2C**) confirming that $\beta3$ integrin is engaged with its ligand in BMP2-induced focal adhesions. The notion that $\beta3$ integrins are involved in cell spreading induced by matrix-bound BMP-2 on soft matrix can be extended to other cell types, as we found that mouse mesenchymal stem cells (D1MSC) respond to bBMP-2 and that this response is impaired by cRGD treatment (**Fig. S2D**). Together these results demonstrate that $\beta3$ integrin needs to be occupied by its ligand to drive cell spreading onto BMP2–soft matrix

in cell lines able to respond to bBMP2. The subsequent question addressed the identity of the ligand of β 3 integrin. Fibronectin, which is one of the β 3 integrin ligands, effectively decorated cell edges, as observed by immunostaining of C2C12 cells plated on low CL films with or without of bBMP-2 (**Fig S3**). Fibronectin could be provided either by the cells or by the serum. The role of serum can be ruled out since cell spreading is still possible onto matrixbound BMP-2 in the absence of serum (**Fig S3A** and in (Crouzier et al., 2011b)). Moreover, the presence of fibronectin around the cells was independent of the presence of serum in the culture medium (**Fig. S3A**). Cells expressed fibronectin as measured by Q-PCR and this expression was not affected by BMP-2 treatment after 4 h of adhesion (**Fig. S3B**). An increase in the fibronectin and collagen mRNA levels became significant only after 30 h of culture (**Fig. S3B**). To test whether β 3 integrin binds to this fibronectin to induce a cell response, C2C12 cells were treated with siRNA against fibronectin. Depletion of fibronectin abolished cell spreading onto BMP2-soft matrix (**Fig.S3C**). These results indicate that $\alpha\nu\beta3$ integrin binds to fibronectin surrounding C2C12 cells to promote BMP-2-induced cell spreading.

Knowing that BMP-2 is able to bind fibronectin through its FN 12-14 domains (Martino et al., 2011, 2014), we tested whether fibronectin might bind matrix-bound BMP2 on soft matrix. Using fluorescence spectroscopy, we showed that a much higher amount of fibronectin can adsorb to the soft films loaded with matrix-bound BMP-2 in comparison to films without BMP-2 (**Fig. S3D**). Of note, although hyaluronan might be involved in cells adhering onto the polyelectrolyte films, our previous data on low and high crosslinked films using either soluble hyaluronan in solution or HA blocking antibodies did not allow us to reveal a specific effect of hyaluronan (Ren et al., 2010).

Our data indicate that, in addition to the transcriptional response, matrix-bound BMP-2 is sufficient and necessary to induce an early mechanical response, e.g C2C12 or mouse mesenschymal stem cell spreading, likely through $\alpha\nu\beta3$ integrin activation. In summary, BMP-2 loaded onto the film is able to provide two anchoring points for cell spreading: one through BMP-2/BMPR interaction for initiating cell spreading and the second through BMP-2/fibronectin/ $\alpha\nu\beta3$ integrin for completing the spreading. These findings support the notion of cooperation between BMP-2 receptors and $\beta3$ integrins on soft films containing matrix-bound BMP-2.



FIGURE 2. β3 integrin is required for cell spreading induced by bBMP-2 on soft films. (A) C2C12 myoblasts plated for 4 h on soft films without BMP-2 (left) or with bBMP-2 (right) were stained for actin and vinculin, indicating the presence of focal adhesions in the case of bBMP-2. (B) C2C12 myoblasts plated for 4 h on soft films without or with Mn2+stimulation were stained for actin and for vinculin. (C) Cells 4 h post-seeding on soft films without BMP-2 or with bBMP-2 were stained for β1, β3, α5, and αV integrin subunits. Insets show zoom-in of the focal adhesions. (D) C2C12 cells were incubated in the absence or presence of β1 and β3 integrin blocking antibodies or (E) depleted in β1 and β3 integrin chains using siRNA strategy before plating on soft films with bBMP-2. (D', E') After 4 h, adherent cell number and spreading area quantified by actin staining significantly decreased in the presence of anti-β3 integrin or after treatment with siRNA against β3 integrin on soft film with bBMP-2. Data are means ± SEM from at least 60 cells per condition. Experiments were performed 3 times. (F) Western blot analysis confirms the efficiency of the siRNA against integrins. *p < 0.05, **p < 0.005. Bar: (A and C) 20 µm; (insets) 5 µm; (D and E) 100 µm.

2.2.c. Matrix-bound BMP-2 increases cell migration by affecting cell adhesion site dynamics

As integrins and BMP-2 in a soluble form have been shown to be involved in cell migration (Dudas et al., 2004; Goldstein et al., 2005; Huttenlocher and Horwitz, 2011; Plotnikov and Waterman, 2013) and as we previously showed that bBMP-2 is involved in cell migration (Crouzier et al., 2011b), we analyzed whether BMP-2 presentation would affect the migration behavior of C2C12 cells by altering adhesion site dynamics. Two approaches were used including time lapse imaging and fluorescence recovery after photobleaching (FRAP) analysis of focal adhesions. Cell tracking assays over 15h confirmed the ability of bBMP-2 to increase cell migration in soft and stiff conditions (velocity of 42 µm/h and 38 µm/h respectively), whereas sBMP-2 did not significantly increase cell migration in both conditions (velocity of 7 µm/h and 17 µm/h respectively) as compared to conditions without BMP-2 (velocity of 6 μ m/h and 19 μ m/h respectively) (Fig. 3A). Moreover, β 3 integrin was shown to be involved as migration speed was decreased by two-fold in case of β 3 integrin deletion (Fig. 3B). These results show that the presentation of BMP-2 by the matrix has a crucial influence on cell migration. Morphological analysis by scanning electron microscopy revealed a marked generation of filopodia in cells on bBMP-2 (Fig. 3C) suggesting a role of BMP-2 in organization of the actin cytoskeleton. We next investigated the possible effects of BMP-2 presentation on focal adhesion dynamics by quantifying the exchange rate of focal adhesion components (Fig 3D) after C2C12 cell transfection of with GFP-paxillin to study single focal adhesions. Cells were plated on stiff films in the presence of sBMP-2 or bBMP-2 in order to enable cell spreading independently of BMP-2 presentation. Our results revealed that the GFP-paxillin recruitment to focal adhesions was 2-fold slower in the case of sBMP-2 as compared to bBMP-2. First, our findings show that matrix-bound BMP-2-induced cell migration is not modulated by substrate stiffness. Secondly, our results suggest that the presentation of BMP-2 by the matrix impacts the dynamics of focal adhesions through a faster recruitment of focal adhesion components such as paxillin.



FIGURE 3. Matrix-bound BMP-2 increases cell migration by affecting focal adhesion dynamics. (A) Tracking experiments of C2C12 cells plated on low or high CL films without BMP-2 or treated with sBMP-2 or bBMP-2. The plotted trajectories of 15 h time-lapse experiments highlight the increased migration speed of cells plated on bBMP-2 whatever the film stiffness. 60 cells per condition are analyzed. (B) C2C12 cells plated on high CL films with bBMP-2 have been monitored in conditions where β 3 integrin was depleted as compared to Si Control. As quantified, the deletion of β 3 integrin abolished this increase of cell migration. 60 cells per condition are analyzed. (C) Scanning electron microscopy Images of C2C12 cells plated on soft film without BMP-2 or treated with bBMP-2. Note the increase of filopodia when cells are subjected to matrix-bound BMP-2. Scale bar: 10 µm. (D) Quantitative measurement of the characteristic recovery time (τ) measured on individual focal adhesion using GFP-paxillin (n=20). The shorter recovery time indicates a higher mobility of GFP-paxillin in the case of C2C12 cells spread onto bBMP-2 in comparison to sBMP-2. *p < 0.05, **p < 0.005

2.2.d. αvβ3 integrin is required to mediate BMP-2 induced Smad signaling pathway through a Src/FAK/ILK/cdc42 axis

As a counterpart to BMP-2 involvement in focal adhesion dynamics, we next investigated whether β 3 integrins are in turn required in a cross-talk for BMP-2 induced Smad signaling. To explore the roles of BMP receptors and integrins in Smad signaling by matrix-bound BMP-2, we examined the Smad response using a luciferase reporter assay and Smad phosphorylation (pSmad1,5, 8^{Cter}) (Fig. 4). As the luciferase signal is increasing as a function of time by displaying a 3-fold higher signal at 8 h and a 6-fold higher signal at 24h than at 4 h, the time point of 15 h was selected for luciferase analysis in order to be able to quantify the effect of drugs or SiRNA (Fig. 4C). As expected, knockdown of BMPR-Ia and of BMPR-II receptors had a strong negative effect on both BMP-2-induced reporter activities and Smad phosphorylation. Strikingly, depletion of β 3 integrins led to a 2- and 3- fold decrease in Smadl phosphorylation at its C-terminus (Fig. 4A, 4B) and in the activity of ID1 promoter in a BMP-responsive element luciferase reporter gene assay (BRE-Luc) respectively (Fig. **4D**), which was not the case for β 1 integrin deletion. The results obtained in the case of β 3 knockdown were similar to those obtained after knockdown of BMP receptors (Fig. 4A, 4B, **4D**). As a control, we used dorsomorphin, an inhibitor of BMP signaling (Yu et al., 2007) known to selectively inhibit BMP type I receptors ActR-I, BMPR-Ia and BMPR-Ib by preventing phosphorylation of Smad proteins (Fig. 4D). Whereas dorsomorphin treatment inhibited Smad phosphorylation as well as luciferase activity, it did not impair cell spreading (Fig. 5B). Thus, our data show that β 3 integrin is required to mediate BMP-2-induced Smad signaling. In addition, our results demonstrate that the phosphorylation of Smad is not involved in β 3 integrin-dependent spreading which is induced by BMP-2. Altogether these results suggest that the spreading is rather due to the BMP-2/BMPR interaction upstream of Smad phosphorylation, identifying β 3 integrin activation as an early event after BMP-2 binding to BMPR. Moreover, the cooperation between BMP receptors and β3 integrins is required for effective Smad signaling in myoblasts in response to matrix-bound BMP-2.

Several transmembrane growth factor receptors, including PDGF and EGF receptors are known to form multiprotein complexes with integrin receptors through Src and FAK, two cytoplasmic kinases associated with cell motility and spreading (Tomar and Schlaepfer, 2010), the adapter ILK (Brakebusch and Fässler, 2003) and RhoGTPase family activity

(Tomar and Schlaepfer, 2009) reinforcing the link between actin cytoskeleton and integringrowth factor receptor complexes (Serrels et al., 2007).



FIGURE 4. β 3 integrins are required to mediate Smad signaling. C2C12 cells were transfected with siRNA against β chain integrins, BMPR-Ia or BMPR-II and plated on soft matrix with bBMP-2 for 4 h. (A) Western Blot and quantification of phospho-Smad1,5,8 (A) and Smad1 (B). Smad pathway activation significantly decreased when cells were transfected with siRNA against β 3 integrin, BMPR-Ia or BMPR-II. (C) Kinetics of luciferase signal in C2C12 cells plated on soft matrix with or without bBMP-2. (D) Analysis of luciferase activity upon dorsomophin treatment or upon deletion of BMP receptors and integrin receptors after 15 h of culture on soft film with bBMP-2. Data are mean <u>+</u> SEM (n=3); NS, not significant; *p < 0.05, ** p < 0.005 compared with siRNA Control.

Therefore, the involvement of the FAK-Src signaling complex in the β 3 integrin/BMP-2 receptor cooperation was explored using a pharmacological approach (**Fig. 5**). Inhibition of Src by PP2 and inhibitor number 5, nb5, of FAK by PF228 and of ILK by Cpd22 all decreased

Smad activity in a dose-dependent manner (Fig. S4A) as measured by the luciferase reporter assay (Fig. 5A) and by Smad phosphorylation (Fig.5C). These results were confirmed using specific siRNA knockdown against Src, FAK and ILK (Fig 5D). Actin filament organization is controlled by the Rho family of small GTPases, including Rho, Rac and Cdc42 (Raftopoulou and Hall, 2004) partly regulated by PI3Kinase (Hanna and El-Sibai, 2013). To explore the mechanical events downstream of BMP-2 stimulation, we evaluated whether perturbations in small GTPase activities or cytoskeleton integrity could affect the Smad response (Fig. 5A and Fig. 5C). PI3Kinase inhibition by LY294002 led to a decrease of luciferase activity (Fig. 5A). Cdc42 inhibition by secramine (Pelish et al., 2006) or by siRNA induced a decrease in Smad activity. In contrast, the Rho inhibitor, Toxin C3, and Rac inhibitor, NSC23766 led to increased Smad signaling (Fig. 5A, Fig 5C and Fig. 5D). To gain more insight into the potential role of the cytoskeleton in bBMP-2-induced Smad signaling on soft films, we employed pharmacological agents known to interfere with cell tension or actin dynamics (Fig. 5A and Fig. 5C). As indicated in Figure 5A and Fig. 5C and 5C', treatment with the ROCK inhibitor (Y27632) or blebbistatin (blebb), which relieves tension on the actin cytoskeleton by inhibiting myosin-II, did not decrease Smad signaling. Interestingly, alteration of actin dynamics with cytochalasin D, an inhibitor that disrupts the actin cytoskeleton by capping filament plus ends, or latrunculin, which disrupts actin cytoskeleton by preventing actin polymerization, reduced luciferase activity by 35 % (Fig. 5A). More importantly, inhibition of LIM kinase (ROCK and Cdc42 effector) by Pyr1 (Prudent et al., 2012), which is important for actin microfilament dynamics, showed a dosedependent decrease of Smad activity down to 60 % (Fig. 5A and Fig. 5C and C' and Fig S4). As bBMP-2 induced spreading, we wondered whether cell shape can regulate Smad signaling. Our data show that the loss of Smad activation induced by Cdc42 inhibition, Src and ILK is correlated with a decrease of cell spreading (Fig. 5A and Fig. 5B). Only FAK inhibition did not follow this trend. In contrast, the absence of Smad phosphorylation by BMPR induced by dorsomorphin treatment had no effect on β 3 integrin-mediated spreading (Fig. 5B). We monitored the step following Smad phosphorylation, which is its nuclear translocation. Smad was already in the nucleus after 4h of spreading onto bBMP-2 on soft matrix (Fig. S4B, 4C, 4D). As a control, we showed that inhibition of Smad signaling using dorsomorphin was associated with a loss of nuclear localization. Consistently with our luciferase assays results, the nuclear localization of pSmad was decreased by about 30%-40% upon Src, FAK or LIMK inhibition whereas it was not affected by ROCK inhibition. As

ROCK inhibition did not affect Smad phosphorylation, ROCK-dependent tension is not directly required for Smad activity in this β 3 integrin/BMPR cross-talk. Our results suggest that instead LIMK-dependent actin dynamics contribute to Smad signaling induced by bBMP-2 on soft matrix.



FIGURE 5. Src, FAK, ILK and Cdc42 but not ROCK mediated BMP-2 signaling induced by bBMP-2. (A) Luciferase activity of p(BRE) luciferase-transfected C2C12 cells was measured after 15 h plating on soft film with bBMP-2 in the presence of various inhibitors of integrin and of Smad signaling (see list in Table SI 2). (grey panel) Inhibitors of Src, FAK, ILK and BMPR receptors: PP2 (Src), nb5 (Src), PF228 (FAK), ILK (Cpd22), (BMPR); dorsomorphin (black panel) inhibitors of RhoGTPases: C3 transferase NSC23766 (Rac), (RhoA), Secramine (Cdc42), Ly294002 (PI3 Kinase); (white panel) Inhibitors of cell cytoskeleton and cell Y27632 (ROCK), blebbistatin tension: (myosin II), cytochalasin D and latrunculin (F-actin), Pyr1 (LIMK). (B) Cell spreading area of C2C12 cells cultured for 4 h on soft films with bBMP-2 in the presence of the same inhibitors (as for A) was quantified. For cell spreading analyses, 60 cells were analyzed per condition, n=3. Data are mean + SEM. The control condition (bBMP-2 on soft films) was normalized to 1 or 100% for luciferase signal and cell spreading, respectively. (C) Effect of inhibitors on Smad1,5,8 phosphorylation. Western blot of phospho-Smad1,5,8 (C, top) and corresponding quantitative analysis (bottom) after cell culture for 4 h on soft films with bBMP-2 in the presence of various inhibitors: dorsomorphin (BMPR), PP2 (Src), PF228 (FAK), Pyr1 (LIMK), Y27632 (ROCK) and

Secramine (Cdc42). (D) Measurement of luciferase activity after 15 h of culture on soft film with matrix-bound BMP-2 upon siRNA treatment against Src, FAK, ILK, ROCK1&2, and Cdc42. The control condition (bBMP-2 on soft films) was normalized to 1 or 100% for luciferase signal and cell spreading, respectively. Data are mean \pm SEM (n=3); *p < 0.05, **p < 0.005 compared with the control condition.

2.2.e. ß3 integrin regulates Smad stability by repressing GSK3 activity

After phosphorylation of Smad at the C terminus by BMPR, the duration of the pSmad1^{Cter} signal is controlled by sequential phosphorylations of the Smad1 linker domain at consensus sites for MAPK and GSK3, which are required for Smad1 proteasomal degradation (Aragón et al., 2011; Fuentealba et al., 2007). GSK3 is negatively regulated by ILK, a downstream effector of β 3 integrins (Delcommenne et al., 1998). As we have previously shown a decrease of pSmad1^{Cter} (Fig. 4A) upon depletion of β 3 integrin, we addressed the question whether β 3 integrin regulates the stability of pSmad1^{Cter} by controlling Smad1 phosphorylation by GSK3 downstream of ILK. First we showed that, after spreading onto matrix-bound BMP-2 on soft matrix, the depletion of β 3 integrin, as opposed to β 1 integrin depletion, totally abolished the phosphorylation of GSK3, demonstrating that GSK3 activity is regulated by β3 integrin (Fig. 6A). Consistently, we found that, after treatment with cycloheximide, an inhibitor of protein synthesis, the duration of Smad signaling was decreased after β 3 integrin depletion (-50%) after 3h) in comparison to control conditions (-50% after 6 h) (Fig 6C) indicating that β 3 integrin-dependent phosphorylation of GSK3 leads to Smad degradation. Downstream of β 3 integrin ILK was also necessary to repress GSK3 activity as judged by the loss of GSK3 phosphorylation upon ILK deletion (Fig. 6A). Strikingly, treatment with a GSK3 inhibitor (SB216763) was able to rescue the luciferase reporter activity after depletion of the downstream effectors of B3 integrins, especially ILK, Src or FAK, pinpointing at GSK3 as their downstream target (Fig. 6B). However, GSK3 inhibitor treatment was not efficient in rescuing the luciferase signal after deletion of β 3 integrin (Fig. 6B) emphasizing an upstream priming role of β 3 integrin in the activation of BMPR. We also noticed that the deletion of BMPR-Ia was more efficient than the deletion of BMPRII in decreasing GSK3 phosphorylation, suggesting an important role of BMPR-Ia in the control of GSK3 by \$\beta3\$ integrin (Fig. 6A).

Our results were extended to mesenchymal stem cells (D1MSC) where the phosphorylation of Smad and GSK3 was also inhibited after β 3 deletion (**Fig. S5C**). Importantly, phosphorylation of GSK3 depended on cell spreading associated with β 3 integrin signaling. Indeed, in conditions where C2C12 cell spreading was imposed by the presence of a stiff substrate (tissue culture polystyrene), cell spreading was not affected by the deletion of β 3 integrin (**Fig. S5A**) but was still associated with a decrease of both Smad (- 30%) and GSK3 (- 50%) phosphorylations (**Fig. S5B**).

Our results demonstrate that β 3 integrin regulates a multi-step process to control Smad activity. First, β 3 integrin is important for assisting BMPR to phosphorylate Smad1 at its C-terminus independently of GSK3 activity. Secondly, β 3 integrin is crucial for the stability of pSmad1^{Cter} by repressing the activity of GSK3 through the downstream Src/FAK/ILK axis.



FIGURE 6. β3 integrin influences BMP-2 signaling through GSK3β inhibition. (A) After depletion of β 1 integrin, β 3 integrin, BMPR-II, BMPR-Ia and ILK, C2C12 cells are spread onto matrix-bound-BMP-2 for 4 h and the level of GSK3 β activity is determined by Western blot analysis by using anti-phosphoGSK3 β antibody. (A, bottom) Quantification of GSK3 β phosphorylation in the different conditions. Data are the mean \pm SEM (n=3). (B) Measurement of luciferase activity at 15 h upon siRNA treatment against β 3 integrin, ILK, Src and FAK with or without GSK3 inhibitor on soft film with bBMP-2. Data are mean \pm SEM(n=3). (C) Monitoring of the life time of Smad1 and phospho-Smad1,5,8 in C2C12 cells spread on TCPS with sBMP-2. C2C12 cells depleted or not with β 3 integrin were incubated with 100 \Box g/ml cycloheximide. Cycloheximide and sBMP-2 were added at t=0. Phospho-Smad1,5,8 and Smad1 protein contents in total lysates were visualized as a function of time from 1 to 18 h by Western blotting. The results are representative of three independent experiments. *p < 0.05 compared with siRNA Control.

2.3. Discussion

In this study, matrix-bound BMP-2 allowed us to dissociate physical and biochemical cues to understand how cells integrate multiple signaling pathways in order to couple cell migration and cell differentiation. First, we demonstrate that matrix-bound BMP-2 is able to initiate a β 3 integrin-dependent mechanical response in a BMPR-dependent and a Smad phosphorylation-independent manner. Second, we identify and give molecular insights into cooperation between β 3 integrin and BMPR for controlling Smad signaling induced by matrix-bound BMP-2. We propose a model wherein β 3 integrin is a key element that acts in a multi-step process by controlling both the primary phosphorylation of Smad1 at its C-terminus by BMPR and the stability of pSmad1^{Cter} through the repression of GSK3 activity (**Fig. 7**).



FIGURE 7: Schematic view of \$3 and BMP-2/Smad integrin/GSK3β cooperation. The interaction between BMP-2 and BMP-2 receptors activates $\alpha v\beta 3$ integrin and mediate cell spreading and cell migration thanks to BMP2/FN interaction. In turn, $\alpha v\beta 3$ is required first to allow the C-terminal phosphorylation of Smad by BMPR and second to inhibit GSK3 activity through Src/FAK/ILK pathway. Both BMP receptors and \$3 integrin signaling converge to control both focal adhesion dynamics and Smad signaling to couple cell migration and fate commitment.

Like many pluripotent mesenchymal cells, C2C12 myoblasts differentiate into distinct lineages depending on the nature of local cues and how they are presented in their

environment. BMP-2 switches C2C12 cell lineage from the myogenic to the osteogenic phenotype (Katagiri et al., 1994b). This osteoblastic lineage commitment in myoblasts is associated with a microenvironmental change that occurs over several days (Ozeki et al., 2007). Our study aimed to decipher the initial steps of BMP-2 response in the osteogenic induction and the involvement of β 3 integrin in Smad signaling.

First, we have shown that matrix-bound BMP-2 through its interaction with BMPR is sufficient to induce the initiation of an adhesive and promigratory phenotype through β 3 integrin clustering, reorganization of the cytoskeleton through stress fibers, filopodia formation and increase of adhesion site dynamics. Our results are in line with previous observations showing the involvement of BMP family in cell migration (Sieber et al., 2009). We have shown that the β 3 integrin-dependent cell spreading is favored by the natural interactions existing between BMP-2 and fibronectin produced by the cells (Martino et al., 2011, 2014). In addition to its interaction with BMPR, BMP-2 bound to the biomaterial is able to interact with fibronectin secreted by C2C12 cells. Consequently, the biomaterial presenting BMP-2 is able to provide two anchorage points for cells: BMP-2/BMPR interaction initiates the formation of focal adhesions containing fibronectin-engaged β 3 integrin. BMP2/fibronectin interaction supports anchorage of β 3 integrin to the biomaterial. The topology of this biomaterial has been essential to optimize the proximity between β 3 integrin and BMPR, hence favoring their cross-talk.

Additionally, we have shown that the role of β 3 integrin upon BMP-2 stimulation is not restricted to cell migration and spreading but is crucial for the initiation of Smad signaling via a multi-step process. The activation of β 3 integrin is the first event of the β 3 integrin/BMPR cross-talk. It requires neither the phosphorylation of Smad at C terminus nor the tyrosine kinase activity of BMPR. Indeed, dorsomorphin treatment upon BMP-2 stimulation inhibits BMPR activity but still preserves β 3 integrin-mediated cell spreading. We have also demonstrated that, in turn, β 3 integrin is necessary for early events in BMP-2 signaling to allow the primary C-terminal phosphorylation of Smad by BMP receptors. The inability of a GSK3 inhibitor to rescue luciferase activity in conditions where β 3 integrin is deleted indicates that the activation of BMPR by β 3 integrin is upstream to the inhibition of GSK3 by β 3 integrin. The use of a biomaterial presenting BMP-2 in a matrix-bound manner has been critical to unveil the involvement of β 3 integrin in the initiation of Smad signaling. Since soft matrix does not sustain high numbers of cell attachment on long times, future experiments will need to test whether this limitation can be rescued with substrate-bound

ligands such as anti-BMPR-Ia. Our study underlines the importance of growth factor presentation such as BMP-2 in a soft context to properly elucidate the molecular mechanisms underlying the perception of biochemical and physical cues of the microenvironment as already described for VEGF (Chen et al., 2010) and EGF (Fan et al., 2007).

Finally, the cooperation between β 3 integrin/GSK3 and BMP-2/Smad pathway highlights the coupling between cell migration and cell-fate commitment. It has been previously shown that GSK3 phosphorylation regulates the duration of Smad signaling (Fuentealba et al., 2007; Sapkota et al., 2007). We demonstrate that GSK3 needs to be negatively controlled upstream by β 3 integrin to modulate Smad phosphorylation and the Smad-associated transcriptional response, which are both important for the osteogenic switch. Consistently, it has been shown that pharmacological inhibition of GSK3 increases the osteogenic propensity of hMSCs cells (Krause et al., 2010). β 3 integrin is not the unique receptor to regulate GSK3 since it has been already described that Wnt, PDGF, FGF signaling can also modulate the GSK3 pathway (Biver et al., 2014; Song et al., 2014). Different cell surface receptors such as N-cadherin (Cheng et al., 1998) or the FGF receptor (Sailer et al., 2005) are also able to modulate BMP-2 responses. This suggests that mechanotransduction-dependent cell commitment results from receptor cooperation to specify the cellular response. It is also likely that both the biochemical and physical properties of the ECM in distinct tissues might dictate the molecular nature of the cluster or the cooperation.

BMP-2 stimulation and osteoblastic lineage commitment in myoblasts are associated with a microenvironmental change that occurs over several days suggesting temporal and contextual effects (Ozeki et al., 2007). In our study, we focused our attention on short term effects (4h to 15h time window) mediated by BMP-2 before osteoblastic switch of C2C12 myoblasts observable after 1 day of BMP-2 stimulation. In light of an elegant series of micropattern experiments showing a relationship between cell shape and cell differentiation (McBeath et al., 2004), we &suspected that cell shape and spreading imposed by β 3 integrin activation might act as an early cue in the commitment process and be responsible for Smad signaling downstream of BMP-2 stimulation. Our data confirm that the spreading mediated by β 3 signaling is induced by BMP-2 stimulation to initiate the Smad response. Whereas the shape-mediated control of osteoblastic lineage specification has been shown to involve cell tension and RhoA/ROCK signaling (Wang et al., 2012), our data demonstrate that the pathway

activated by matrix-bound BMP-2 in myoblast cells leading to early Smad driventranscription is dependent on Cdc42/LIMK and independent of Rho/ROCK activation. While 4-8h might not be sufficient time for significantly elevated transcription versus control (Fig.4C), the luciferase construct might not have the same kinetics as SMAD targets in the genome, although non-canonical pathways remain an alternative. This discrepancy may be explained by the time scale difference in BMP-2 stimulation and/or by differences in BMP-2 presentation: short-term for BMP-2 presented from the soft matrix (4-15 h) in the present study as compared to longer-term BMP-2 stimulation (2 days) for cells plated on a stiff micropatterned substrate with sBMP-2 (Wang et al., 2012). BMPR/β3 integrin cross-talk is likely to be relevant for the establishment of a transient new phenotype before the conversion from myoblasts to osteoblasts. Our results suggest that this conversion starts with Cdc42/LIMK pathway activation under the control of a cross-talk between β3 integrin and BMP receptors. However our observations derived from a soft matrix-bound BMP-2 are in line with the suppression of RhoA activity in compliant settings (Engler et al., 2006) and the ability of LIMK not only to interact with BMPRII (Foletta et al., 2003) but also to be activated via Cdc42/FAK pathway independently of ROCK pathway in myoblast cells (Gamell et al., 2008). Like physical cues (Dingal et al., 2015; Engler et al., 2006; Swift et al., 2013), our data show that, BMP-2 as a biochemical cue, is able to induce cytoskeletal reorganization that precedes the osteogenic switch. The involvement of β 3 integrin and LIMK in the control of the phosphorylation of cofilin might support the need for temporal control of actin turnover, the necessity of continuous repression of actin depolymerization or its participation in the formation of actin/cofilin rods important to initiate or support osteogenic program (Dopie et al., 2012; Munsie et al., 2012; Sen et al., 2015). Our results do not exclude the involvement of the ROCK pathway and its control by another integrin at later stages of myoblast-osteoblast differentiation switch. Our results lead to the intriguing but intuitive idea that different integrins might have somewhat opposing or rather complementary mechanical roles during the time window of muscle-osteogenic trans-differentiation. Given the increase of ECM stiffness in osteoblastic lineage which can be mimicked by a plastic substrate (McBeath et al., 2004), β1 integrin /ROCK signaling might substitute for β3 integrin/LIMK signaling later on during ECM stiffening upon the myoblast/osteoblast switch imposed by BMP-2 stimulation.
In a broader perspective, this coupling between integrins and BMPR signaling pathways is of great relevance in developmental processes and regenerative medicine, where cell recruitment is a prerequisite before cell differentiation to form a specific organ or repair damaged tissue. Identification of signaling pathways such as the β 3 integrin-GSK3 axis here and tools to control β 3 integrin and GSK3 activities via engineered biomaterials and/or pharmacological agents would provide new therapeutic strategies for optimizing bone repair and regeneration.

2.4. Materials and methods

Buildup of (PLL/HA) films, cross-linking and loading of rhBMP-2

HA (sodium hyaluronate, $2x10^5$ g/mol) was purchased from Lifecore Biomedical (USA) and PLL ($2x10^4$ g/mol) was purchased from Sigma (France). PLL (0.5 mg/mL) and HA (1 mg/mL) were dissolved in a Hepes-NaCl buffer (20 mM Hepes pH 7.4, 0.15 M NaCl). For all experiments, (PLL/HA)₁₂ films (ie made of 12 layer pairs of PLL and HA) were prepared as previously described (Crouzier et al., 2009) with a dipping machine (Dipping Robot DR3, Kierstein GmbH, Germany) on 14 mm or 32 mm diameter glass slides (VWR Scientific, France). For quantification of cell adhesion, the films were manually constructed in 96-well plates (Nunc, Denmark) starting with a first layer of poly(ethyleneimine) ($7x10^4$ g/mol, Sigma, France) at 3 mg/mL. Briefly, polyelectrolyte solutions (50μ L) were deposited in each well, left to adsorb for 8 min, before being washed twice with rinsing solution (100 μ L of 0.15 M NaCl, pH~6) for 1 min. The sequence was repeated until the buildup of a (PLL/HA)₁₂ film was achieved. The films were cross-linked following the protocol previously described using 1-Ethyl-3-(3-Dimethylamino-propyl)Carbodiimide (EDC) at 30 (soft films) or 70 mg/mL (stiff films) and *N*-Hydrosulfosuccinimide (sulfo-NHS) at 11 mg/mL (both purchased from Sigma, France) (Crouzier et al., 2009)

The BMP-2 (Clinical Grade, Medtronic, France) was incorporated into films pre-equilibrated for 30 min in the medium in which BMP-2 was suspended (1 mM HCl). It was deposited onto the films and left to adsorb overnight at 4°C. The coated slides were thoroughly washed for 1 h in Hepes-NaCl in order to keep only matrix-bound BMP-2 (Crouzier et al., 2009) before being sterilized for 15 min under UV light. The experiments were carried out at least 3 times, with at least three triplicate samples per condition in each experiment.

Mechanical properties of the films were characterized by nanoindentation with a colloidal probe using atomic force microscopy as described previously (Boudou et al., 2011). The force/indentation curves were fitted by a modified Hertz model to take into account the finite film thickness.

Cells and reagents

C2C12 cells and mesenchymal stem cells (D1MSC) (ATCC, < 20 passages) were maintained in polystyrene flasks in a 37°C, 5% CO₂ incubator, and cultured in a 1:1 Dulbecco's Modified Eagle Medium (DMEM)/F12 medium and α -MEM respectively (Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France), containing 10 U/mL penicillin G and 10 µg/mL streptomycin (Gibco, Invitrogen, Cergy-Pontoise, France) (growth medium, GM). Cells were subcultured prior to reaching 60-70% confluence (approximately every 2 days). For all experiments, C2C12 cells seeded on films at 1.5×10^4 cells/cm² in growth medium were allowed to grow for 4 h. A full list of the inhibitors used as well as their working concentration can be found in Table S2. After dissolution in DMSO, they were added into the medium at the same time as cell plating on films.

Hamster anti- α_5 , rat anti- β_1 and hamster anti- β_3 integrin blocking antibodies were purchased from BD Biosciences (Pont de Claix, France). Hamster anti-av blocking antibody was purchased from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France). For immuno-fluorescence, anti- α_5 and rat anti- β_1 were purchased from Chemicon (Millipore SAS, Molsheim, France). Hamster anti- α v and hamster anti- β 3 were purchased from Santa Cruz Biotechnology. Phalloidin-tetramethyl rhodamine isothiocyanate (TRITC), mouse antivinculin and rabbit anti-fibronectin were purchased from Sigma (St Quentin Fallavier, France); rabbit) anti-Smad1 and rabbit anti-pSmad1,5,8 were from Cell Signaling. AlexaFluor-conjugated secondary antibodies and Hoechst 33342 were purchased from Invitrogen. 3,3'-dithiobis-sulfosuccinimidylpropionate (DTSSP) was from Pierce (Thermo Fischer Scientific, Brebières, France). Antibodies used for Western blotting were rabbit anti-Smad1 (Cell Signaling) rabbit anti-phosphoSmad1,5,8 (Cell Signaling), rabbit anti-GADPH (Cell Signaling), rabbit anti-phospo-GSK3ß (Cell signaling), home-made rabbit anti-ß1 integrin (Albiges-Rizo laboratory), rat anti-\beta3 integrin (Emfret Analytics, Eibelstadt, Germany) and mouse anti-actin (Sigma). Cyclic RGD peptide and negative control cRAD were purchased from Anaspec (Tebu-Bio, Le Perray en Yvelines, France).

Quantification of cell adhesion and integrin binding assays

The cell counting tests were performed in 96-well plates. The cell numbers were assessed after 4 h of adhesion using a cell counting kit (CyQUANT, Molecular Probes, Invitrogen, France). In brief, the cells were washed three times with phosphate buffered saline (PBS) and frozen at -80 °C overnight. After thawing the cells at room temperature (RT), a mixture of the CyQUANT GR dye and cell-lysis buffer was introduced and the fluorescence of the plates was measured using the Tecan Infinite 1000 spectrofluorimeter (Ex 485/ Em 535) (Tecan, Austria). For inhibition of initial adhesion by anti-integrin antibodies, cells (10⁵ cells/mL) were pre-treated with either anti- β or anti- α integrin subunits at 10 µg/mL for 30 min at 37°C. The cells in the presence of antibodies were then seeded onto the surfaces at 10⁴ cells/cm². For integrin activation with Mn²⁺, MnCl₂ at 0.5 mM was directly added to the cell suspension during the adhesion phase for 1 or 4 h (Cluzel et al., 2005).

Immunofluorescence

For staining of F-actin, vinculin, fibronectin, Smad1, and pSmad1,5,8, cells were fixed in 3.7% formaldehyde in PBS for 20 min and permeabilised for 4 min in TBS (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.2% Triton X-100. After rinsing with PBS, samples were incubated for 1 h in 0.1 % BSA in TRIS-buffered saline (50 mM TRIS, 150 mM NaCl, 0.1% NaN₃, pH 7.4). Actin was labeled with phalloidin-TRITC for 30 min. Cell nuclei were stained with 5 μ g/mL of Hoechst 33342 for 10 min. After the incubations with the primary antibodies (diluted in 0.2% TBS-gelatin) for 30 min at RT, cells were washed 3 times in TBS and incubated for 30 min with the secondary antibodies.

For α_5 and α_V , β_1 and β_3 integrin staining, a protocol adapted from that of Garcia *et al.* (Keselowsky et al., 2005) was employed. Briefly, cells were rinsed in PBS and incubated in ice-cold DTSSP (in 1 mM final concentration in PBS) for 30 min. Unreacted cross-linker was quenched with 50 mM Tris in PBS for 15 min and bulk cellular components were extracted in 0.1% SDS in PBS. The slides were then blocked in 0.1 % BSA in TBS. After this, bound integrins were immunostained with antibodies against α or β chains and Alexa-Fluor 488-conjugated secondary antibody. All the slides were mounted onto coverslips with antifade reagent (Prolong, Invitrogen) and viewed under fluorescence microscopy (LSM 710, Axiovert 200M, Zeiss, Germany) using a 10X/0.25NA, 20X/0.8NA or 63X/1.4NA objectives. Images were acquired with Metaview software using a CoolSNAP EZ CCD

camera (Ropper Scientific, Evry, France). To quantify cell spreading, fluorescence images were analyzed with the ImageJ software to determine average cell area.

SiRNA interference

Cells were transfected with siRNA against β_1 or β_3 integrins, BMP receptor Ia or II, Src, FAK, ILK, Cdc42, ROCK1&2, FN (ON-TARGET plus SMARTpool, respectively Mouse ITGB1, ITGB3, BMPR-Ia and BMPR-II, Src, Ptk2, Cdc42, ILK, Rock1 and Rock2, FN Thermo Scientific Dharmacon). The gene target SiRNA sequences used for transfection are listed in Table S3. A scrambled siRNA (all stars negative control siRNA, Qiagen) was taken as control. Cells were seeded at 5 000 cells/cm² in 6-well plates and cultured in 2 mL of GM for 15 h. The transfection mix was prepared as following. For one well, 6 μ L of Lipofectamine RNAiMAX Reagent (Invitrogen) were added to 305 μ L of Opti-MEM medium. Lipofectamine-containing mix was added to siRNA-containing mix and incubated for 20 min at RT. Prior transfection, the GM of the wells was replaced by the GM without antibiotics. Then, 610 μ L of the final mix were added to each well. After 24 h of incubation at 37°C, the cells were transfected for the second time following the protocol described above and incubated for another 24 h. Then the cells were detached by trypsin-EDTA, seeded in GM at 15 000 cells/cm² on the films and allowed to adhere for 4 h.

Smad assay using Luciferase reporter gene

C2C12 stably transfected with an expression construct (BRE-Luc) containing a BMPresponsive element fused to the firefly luciferase reporter gene (Logeart-Avramoglou et al., 2006) were used (generous gift from D. Logeart-Avramoglou, Paris Diderot University). They were cultured under the same conditions as non-transfected C2C12 cells. After 15 h of culture on the films, cell lysis and luciferase measurements were carried out according to the manufacturer's instructions (Bright-GloTM luciferase assay system, Promega). As the luciferase signal is increasing as a function of time by displaying a 6-fold higher signal at 24 h than at 4 h (250 versus 1600 arbitrary units), the time point of 15h was selected for luciferase treatment in order to be able to quantify the effect of drugs or SiRNA.

Measurements were normalized to the DNA content of each sample as measured by the cyQUANT assay. The effect on various drugs on BMP pathway was assessed in 96-well plates using 15 000 cells/cm² and drugs at various concentrations.

Measurement of ALP activity in C2C12

After 3 days in culture on BMP-2 loaded films in 96 well plates, C2C12 cells were assayed for alkaline phosphatase activity (ALP), a marker for osteoblast differentiation. After removal of culture medium, cells were lysed by adding a 50 μ L of 0.5% Triton-X100 in PBS supplemented with 0.15 μ l benzonase (Novagen) and 1x antiprotease (anti-protease complete; Roche, Germany). The plate was put at 37°C for 20 min and half of the volume was conserved for protein assay. A buffer containing 0.1 M 2-amino-2-methyl-l-propanol (Sigma, St Quentin-Fallavier, France), 1 mM MgCI₂, 9 mM *p*-nitrophenyl phosphate (pNPP) (Euromedex, Mundolsheim, France), adjusted to pH 10 with HCl was used to assay the cell lysate for ALP. Reaction was followed over 5 min in a 96-well plate by measuring the absorbance at 405 nm using a Tecan Infinite 1000 Microplate reader. The activity was expressed as μ moles of p-nitrophenol produced per minute per mg of protein. Total protein contents of the samples were determined using a BCA protein assay kit (Interchim, Montluçon, France).

Immuno-blotting

Cells were lysed in Laemmli buffer. Detection of proteins by Western blotting was done according to standard protocols. After electrotransfer and blocking (10 mM Tris, pH 7.9, 150 mM NaCl, 0.5% Tween 20, 3% dry milk at room temperature for 1 h), the PVDF membrane was incubated with antibodies overnight at 4°C. Immunological detection was achieved with HRP-conjugated secondary antibody. Peroxidase activity was visualized by ECL (West pico signal, Pierce) using a ChemiDoc MP imaging system (Bio-Rad). Densitometric quantification of the bands was performed using the Image Lab program (Bio-Rad). As control, detection of actin was also performed.

qPCR

RNA reverse transcription and real time quantitative PCR Total RNA was prepared from C2C12 myoblasts after cell lysis using a kit (Zymo research, Proteigene, France). After reverse transcription of 1 μ g total RNA, PCR was carried out using a set of gene specific primers for fibronectin and collagen. The sequences of primers used for real time PCR are listed in Table S4. cDNA (equivalent to 10 ng) was used for real time quantitative PCR, performed with a thermocycler MX4800P (Stratagène). The 12 μ L reaction mix contained 1 μ L of Master SYBR Green I mix, including Taq DNA polymerase, buffer, deoxynucleoside

trisphosphate mix, SYBR Green I dye, 3 mM MgCl₂ and 0.5 μ M of each primer. 2 μ L of 30fold diluted cDNA was added to the mixture. Primer efficiency was established by a standard curve using sequential dilutions of gene specific PCR fragments. Data were normalized from RT-QPCR housekeeping gene ATP50 as an index of cDNA content after reverse transcription.

Time lapse image acquisition

Cells were plated on either low or high CL films without BMP-2 or with sBMP-2 or bBMP-2 in standard growth medium at 37°C in 5% CO₂. Time lapse images were acquired every 15 min over a 16 h period (after the initial 4 h adhesion period) using a 10x/0.3NAobjective in phase contrast microscopy (Axiovert 200M, Zeiss, Germany). Images were acquired with Metaview software using a CoolSNAP HQ2 CCD camera (Ropper Scientific, Evry, France). Migration velocities were measured using "Manual Tracking" and "Chemotaxis and Migration Tool" plugins from ImageJ. For both analyses, at least 60 cells were analyzed for each time point.

FRAP experiment

C2C12 transfected with EGFP-paxillin were cultured on stiff film with sBMP-2 or bBMP-2 12 hours before experiment. FRAP experiments were performed in standard growth medium at 37°C in 5% CO₂. with a confocal microscope (LSM710, Zeiss, Germany) using 63X objective (Plan-Apochromat 63x/1,4 Oil DICIII, WD 190) equipped with on-stage incubator. One 0.03 μ m² square area located in the center of one focal adhesion was processed by FRAP. EGFP fluorescence in this adhesion area was eliminates by 10 bleach cycles at 100% intensity of the 488 nm argon laser. The fluorescence recovery was then sampled with low laser power (3%) each 5 seconds for 3 min. The recovery curves were obtained using Zen software. The corrected curve was adjusted with origin software using monoexponential fit. The characteristic recovery time (τ) of EGFP-paxillin deduced from the fit of the experimental data was the mean of at least 20 individuals FAs.

Scanning electron microscopy imaging

Cells grown on low CL films for 4 h were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate/0.1 M sucrose, pH 7.2. Samples were then gradually dehydrated using increasing concentrations of ethanol mixtures up to 100%. Before imaging, the samples were air-dried

and then carbon-coated. The samples were observed using a Quanta 250 Field emission gun (FEG) SEM (FEI Company) at 5 keV equipped with a high contrast backscatter detector.

Lifetime of Smad1 and phospho-Smad1,5,8 measurement

C2C12 cells depleted or not with β 3 integrin were seeded at 30 000 cells/cm² in 6-well plates. After 4 h (t=0), cells were incubated with 100 µg/ml cycloheximide and 600 ng/mL BMP-2. After indicated time, cells were lysed and the phospho-Smad1,5,8 and Smad1 protein content in the total lysates was visualized by Western blotting

Statistical analysis

Error bars represent standard errors and statistical analysis was performed using Sigma-Plot v12.5 software. Student t-test has been used to evaluate the statistical differences between two samples. In case of cell spreading and FRAP experiments, Mann Whitney rank sum test has been used. Statistical significance was determined at α =0.05. NS, not significant; *p < 0.05, ** p < 0.005.

Online supplemental material

Fig. S1 shows the increase of BMP-2 potency when bound to matrix. Fig. S2 shows how Low CL film without BMP-2 provides inadequate adhesion for C2C12 cells and identification of αv integrins as important receptors to mediate BMP-2-induced cell spreading. Fig. S3 shows that bBMP-2 does not induce the expression of fibronectin and collagen but cell spreading depends on bBMP2 and fibronectin. Fig. S4 shows the control of nuclear localization of Smad by Src, FAK and LIMK. Fig. S5 shows the involvement of $\beta 3$ integrin and BMPR in Smad and GSK3 phosphorylations in both C2C12 and D1 MSC cells. Table S1 shows the physico-chemical properties of (PLL/HA) films loaded or not with bBMP-2. Table S2 gives the list of inhibitors used to interfere with BMPR receptor and $\beta 3$ integrin signaling, their working concentration and provider. Table S3 gives the list of the gene target SiRNA sequences used for transfection. Table S4 gives the primer sequences used for qPCR experiments.

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2.5. Supplementary material



FIGURE S1. BMP-2 potency is increased when bound to matrix. (A) The biomaterial for the study of BMP-2 effects on cell behavior combines two functional properties of the tunable multilayer film system. (1) A polyelectrolyte multilayer film is built onto a substrate by alternating deposits of two polyelectrolytes: poly(L-lysine) and hyaluronan (PLL/HA). (2) The film can be covalently crosslinked to different levels using a water soluble carbodiimide, thus allowing film stiffness to be modulated. (3) BMP-2 is loaded into the film. BMP-2 is trapped in the film and delivered to the cells in a "matrix-bound" manner. (B) Luciferase activity of p(BRE) luciferase-transfected C2C12 cells was measured after 15 h of plating on tissue culture plastic (TCPS), soft PLL/HA film (Low CL) or PLL/HA stiff film (High CL) in the presence of soluble (sBMP-2) or matrix-bound BMP-2 (bBMP-2). (C) Western blot analysis of Smad activation using an anti-phospho Smad1,5,8 antibody. The analysis of the intensity (ratio of phospho-Smad1,5,8/Actin) shows that phosphorylation of Smad occurs only when BMP-2 is present. (D) ALP expression after 4 days on soft and stiff films without/with BMP-2 shows that the signal is similar to that of soluble BMP-2 or higher in the case of the soft film. Experiments in (B, C, D) have been performed 3 times. NS, not significant; *p < 0.05, ** p < 0.005



FIGURE S2: av integrins are required to mediate BMP-2-induced spreading. (A) Low CL film without BMP-2 provides inadequate adhesion for C2C12 cells. C2C12 cell spreading on low CL films with bBMP-2 can be maintained for at least 24 h. At longer times, these cells tend to form cellular clusters and cohesive aggregates, which may be related to their osteogenic differentiation. (B) C2C12 cells were treated with blocking antibodies against α 5 and α v chain integrins and plated for 4 h on low CL film with bBMP-2. After 4 h, the number of adherent cells and cell spreading were quantified after labeling the actin cytoskeleton. The number of adherent cells and cell spreading area significantly decreased after treatment with antibodies against α v integrin on soft matrix-bound BMP-2. (C) C2C12 cell adhesion on low CL film with bBMP-2 and on glass in the presence of cRAD and cRGD peptides in comparison to the control condition. Note the loss of C2C12 spreading upon treatment with cRGD. (D) D1 MSC adhesion onto low CL film in the absence or presence of bBMP-2. D1 MSC cell spreading is blocked in the presence of cRGD. Data are means ± SEM from at least 60 cells per condition. Experiments were performed 3 times. ** p < 0.005 compared with control conditions. Scale bar: 100 µm.



FIGURE S3: BMP-2-bound matrix does not induce the expression of fibronectin and collagen but cell spreading depends on bBMP2 and fibronectin. (A) Immunostaining of fibronectin in cells grown on low CL film without or with bBMP-2 for 4 h, in presence or absence of serum. (B) qPCR of fibronectin and collagen mRNA expression shown over 72 h. (C) C2C12 cells were treated with SiRNA against fibronectin and plated onto low CL film with bBMP-2 before quantifying the relative cell area. The deletion of fibronectin is controlled by western blot. Data are means \pm SEM from at least 60 cells per condition. (D) Fibronectin is pre-adsorbed or not on the low CL films without or with bBMP-2 and quantified by fluorescence spectroscopy after immunolabelling of the adsorbed fibronectin. Data are means \pm SEM (n=4); ** p < 0.005. Scale bar: 100 µm.



FIGURE S4: Nuclear localization of Smad is controlled by Src, FAK and LIMK. (A) Dose-dependent effect of the inhibitors against Src, FAK, Cdc42, LIMK and ILK. Luciferase activity of p(BRE) luciferase-transfected C2C12 cells was measured after 15 h plating on soft film with bBMP-2 in the presence of inhibitors with different concentrations. Data are means \pm SEM (n=4) (B) Nuclear localization of Smad1 after 4h on matrix-bound BMP-2 was observed by immunostaining in the presence of a series of inhibitors (dorsomophin, PP2 (Src), PF228 (FAK), Pyr1 (LIMK) and Y27632 (ROCK)) as compared to control condition (DMSO); upper raw: anti-Smad1, lower raw: DAPI. (C) Quantification of nuclear Smad1 and pSmad 1,5,8 in absence or presence of inhibitors. Data are means \pm SEM from at least 60 cells per condition. NS, not significant; *p < 0.05, ** p < 0.005 as compared to control. Scale bar: 20 µm.

Chapitre II. Coopération entre l'intégrine β 3 et les récepteurs BMP dans l'étalement cellulaire et la réponse SMAD



FIGURE S5. Smad and GSK3 phosphorylations are dependent on β 3 integrin and BMPR in both C2C12 and D1 MSC cells. (A) Bright field images of C2C12 cells after siRNA against β 1integrin, β 3 integrin, BMPR-Ia and BMPR-II spread onto tissue culture polystyrene in presence of sBMP-2 for 4 hours. C2C12 cells (B) or D1 MSC cells (C) are spread onto tissue culture polystyrene in presence of sBMP-2 for 4 hours after depletion of β 1 integrin, β 3 integrin, BMPR-Ia, and BMPR-II in order to analyze by western blot the level of Smad and GSK-3 β activities using anti-phospho-Smad1,5,8 and phospho-GSK3 β antibodies. Data are means \pm SEM (n=3)). The efficiency of β 1 and β 3 integrin deletion is validated by western blot as shown in the lower panel. *p < 0.05, ** p < 0.005 as compared to control. Scale bar: 100 μ m.

Film stiffness E ₀ (kPa)	No BMP-2	Matrix-bound BMP-2
Low cross-linker (soft films)	182 ± 20	213 ± 34
High cross-linker (stiff films)	358 ± 48	367 ± 58
Contact Angle (degree)		
Soft films	43 ± 4	48 ± 4
Stiff films	48 ± 4	55 ± 7
Mean roughness (nm)		
Soft films 4.2 ± 0.8		4.8 ± 0.2
Stiff films	6.8 ± 1.0	7.9 ± 0.4

Table S1.	Physico-cher	nical chara	cteristics o	of soft and	stiff films	without or	with	bBMP-2
								~

. Physical chemical characteristics of soft or stiff (PLL/HA)₁₂ films without BMP-2 or in the presence of BMP-2 loaded in the films from a solution of 20 μ g/mL. Film stiffness, contact angle and film roughness were statistically similar without or with bBMP-2 (p > 0.05).

Target protein	Inhibitor	Manufacturer	Working Concentration
LIMK	Pyr1	Prudent et al, Cancer Res. 2012	25 μΜ
ROCK	Y27632	Calbiochem	10 µM
Rac	NSC 23766	Calbiochem	100 µM
MLC	Bebblistatin	Calbiochem	5 μΜ
Rho A	C3 transferase	Cytoskeleton	17 µM
BMPR/AMPK	Dorsomorphin	Calbiochem	10 µM
Src	PP2	Sigma	25 µM
Src	Number 5	Biaffin	10 µM
FAK	PF 228	Calbiochem	10 µM
Cdc42	Secramine	Pelish et al, Nat. Chem. Biol. 2006	5 μΜ
PI3K	LY294002	Tocris	25 µM
ILK	Cpd22	Calbiochem	2 µM
GSK3β	SB 216763	Tocris	10 µM
F-actin	Cytochalasine D	Calbiochem	2 µM
F-actin	Latrunculin	Calbiochem	5 µM

Table S2. List of all pharmacological inhibitors and concentrations used

Target gene	Reference	siRNA target sequences
BMPR-Ia	ON-TARGET plus SMARTpool, Mouse BMPR-Ia Thermo Scientific Dharmacon L-040598-00-0005	GAGGAAUCGUGGAGGAAUA GCUAGCUGGUUUAGAGAAA GAAAUGGCUCGUCGUUGUA GGCCAUUGCUUUGCCAUUA
BMPR-II	ON-TARGET plus SMARTpool, Mouse BMPR-II Thermo Scientific Dharmacon L-040599-00-0005	GCACAUAGGUCCCAAGAAA GAACGCAACCUGUCACAUA GCAUGAACCUUUACUGAGA CUAAUAAGCUAGAUCCAAA
β1 integrin	ON-TARGET plus SMARTpool, Mouse ITGB1Thermo Scientific Dharmacon L-040783-01-0005	UGCCAAAUCUUGCGGAGAA UUACAAGAGUGCCGUGACA GUGAAGACAUGGACGCUUA CAAUGAAGCUAUCGUGCAU
β3 integrin	ON-TARGET plus SMARTpool, Mouse ITGB3Thermo Scientific Dharmacon L-040746-01-0005	AAACAGAGCGUGUCCCGUA AAACACGUGCUGACGCUAA GAGCAGUCUUUCACUAUCA GUGAAAGAGCUGACGGAUA
Src	ON-TARGET plus SMARTpool, Mouse Src Thermo Scientific Dharmacon L-040877-00-0005	GCACGGGACAGACCGGUUA GGGAGCGGCUGCAGAUUGU UCAGAUCGCUUCAGGCAUG GCUCGUGGCUUACUACUCC
FAK	ON-TARGET plus SMARTpool, Mouse Ptk2 Thermo Scientific Dharmacon L-041099-00-0005	GAAGUUGGGUUGUUUGGAA GGGCAUCAUUCAGAAGAUA GCUCCAGAGUCAAUCAAUU GUACAGCACUCGCGUAUCU
Ilk	ON-TARGET plus SMARTpool, Mouse ILK Thermo Scientific Dharmacon L-040115-00-0005	GGACAUUGCACAAGGCCUA GCGCUUACAGAGUAUAUGA GAAGAUUCCUGUGUAUAGG GAACUUUGGUGGGAAAUGA
Cdc42	ON-TARGET plus SMARTpool, Mouse Cdc42 Thermo Scientific Dharmacon L-043087-00-0005	GACUACGACCGCUAAGUUA CGGAAUAUGUACCAACUGU GAUGACCCCUCUACUAUUG AAACAGAAGCCUAUUACUC
Rock1	ON-TARGET plus SMARTpool, Mouse Rock1 Thermo Scientific Dharmacon L-046504-00-0005	GCACCAAUCUAUCGAAGAG UGUCGAAGAUGCCAUGUUA GACCUUCAAGCACGAAUUA GCGGUUAGAACAAGAAGUA
Rock2	ON-TARGET plus SMARTpool, Mouse Rock2 Thermo Scientific Dharmacon L-040429-00-0005	GAGAUUACCUUACGGAAAA GCAAUGAAGCUUCUUAGUA CACAACAGAUGAUCAAAUA GGACAUGAGUUUAUUCCUA
Fibronectin	ON-TARGET plus SMARTpool, Mouse FN Thermo Scientific Dharmacon L-043446-01-0005	AGAACAAACACUAACGUAA GGUCAUUUCAGAUGCGAUU GGAGAGAGAUGCACCGAUU GGUUCAGACUCGAGGCGGA

Table S3. List of the gene target SiRNA sequences used for transfection

Gene target	Primer sequences		
BMPR-Ia	For	TGCAAGGATTCACCGAAA	
	Rev	TGCTGCCATCAAAGAACG	
BMPR-II	For	TTGGGATAGGTGAGAGTCGAAT	
	Rev	TGTTTCACAAGATTGATGTCCCC	
Fibronectin	For	GCAGTGACCACCATTCCTG	
	Rev	GGTAGCCAGTGAGCTGAACAC	
Collagen	For	TGT GTG CGA TGA CGT GCA AT	
	Rev	GGG TCC CTC GAC TCC TAC A	
ATP50	For	CTATGCAACCGCCCTGTACT	
	Rev	GATGATACCCTGGGTGTTGC	

 Table S4. List of primer sequences for BMP receptors, fibronectin and collagen as well as for ATP50

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2

Cette deuxième partie fait l'objet d'un article qui sera soumis dans Cell Death and Differentiation

1. Résumé

1.1. Introduction

Nous avons montré précédemment que les récepteurs BMP-2 et l'intégrine β3 coopéraient et convergeaient pour lier la migration cellulaire et l'induction de la différenciation en contrôlant les étapes précoces de l'étalement cellulaire et de la signalisation Smad (Fourel et al., 2016). Pour obtenir une architecture tissulaire correcte durant la morphogenèse, les cellules doivent interagir les unes avec les autres et avec la matrice extracellulaire (MEC). Ces interactions sont médiées par deux classes de récepteurs d'adhésion : les intégrines et les cadhérines qui sont mécaniquement interconnectées pour conduire à la formation du tissu et au maintien de l'intégrité tissulaire (Berrier and Yamada, 2007; Chen and Gumbiner, 2006; Mui et al., 2016; Weber et al., 2011). Etant donné les changements des propriétés mécaniques de la MEC pendant la fenêtre temporelle de transdifférenciation MUSCLE/OS, nous nous sommes intéressés aux processus par lesquels la BMP-2 opère un changement dans la machinerie adhésive pour une adaptation au nouveau microenvironnement et conduire à la différenciation cellulaire.

1.2. Résultats

L'analyse des cinétiques d'expression génique (en RT-qPCR) des facteurs de transcription MUSCLE (MyoD et Myogenin) et OS (Osterix) et de leur translocation au noyau (en Immunofluorescence) a validé la capacité de la BMP-2 chargée dans les films de (PLL/HA) à induire la transdifférenciation MUSCLE/OS des myoblastes C2C12. Nous avons mis en évidence un changement de matrice durant cette transdifférenciation, les cellules exprimant davantage la Fibronectine et le Collagène I et remodelant différemment la Fibronectine lorsqu'elles sont en présence de BMP-2, quelque soit son mode de présentation. De plus, des analyses en RT-qPCR et Western Blot ont montré que ce changement matriciel est associé à une adaptation du répertoire de récepteurs d'adhésion cellule/matrice (intégrines) et cellule/cellule (cadhérines). Tandis que les C2C12, en différenciation musculaire, expriment la BMP-2 engendre une augmentation de l'expression de la cad-11 et des intégrines spécifiques du Collagène.

Notre étude révèle que des couplages intégrine/cadhérine contrôlent l'ostéogenèse induite par la BMP-2. L'analyse de la réponse à la BMP-2 après déplétion des intégrines et cadhérines a montré que ITGB3 et ITGB5 coopèrent avec les cadhérines N et 11 pour contrôler l'activité transcriptionnelle, tandis que ITGB1 et cad-11 sont toutes les deux impliquées dans l'organisation de la matrice extracellulaire et la formation tissulaire.

2. Article

Interplay between integrins and cadherins to control bone differentiation upon BMP-2 stimulation

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ABSTRACT:

Upon BMP-2 stimulation, the osteoblastic lineage commitment in C2C12 myoblasts is associated with a microenvironmental change that occurs over several days. Herein we addressed the question as to whether BMP-2 could operate a switch in adhesive machineries to adapt to the new microenvironment and to drive bone cell fate decision. Our findings highlight the switch of integrin and cadherin expression during muscle-bone transdifferentiation upon BMP-2 stimulation whatever its mode of presentation. Whereas C2C12 muscle cells express M-cadherin and Laminin specific integrins, the BMP-2 induced transdifferentiation into bone cells is associated with an increase in the expression of cadherin-11 and Collagen-specific integrins. Our study reveals how integrins and cadherins work in a combinatorial fashion and exert distinct functions to drive the osteogenic program

and to control bone cell plasticity. In bone cells, whereas β 3 integrin and β 5 integrin are working with N-cadherin to control transcriptional activities, integrin β 1 and cadherin-11 are dedicated to cell mechanics by both shaping octagonal cells and organizing the extracellular matrix. Our results show that different sets of integrins and cadherins may split the job by having somewhat complementary mechanical roles during the time window of muscleosteogenic trans-differentiation.

2.1. Introduction

The functional interactions between muscle and bone occur through growth factors and cytokines and allow for inter-organ communication that is both required to maintain tissue homeostasis and critical during bone tissue regeneration (Glass et al., 2011; Rosen, 2011; Tsuji et al., 2006; Yu et al., 2010b). Following bone injury, muscle-derived stem cells have been shown to be activated during the inflammatory phase of repair (Abou-Khalil et al., 2014; Colnot et al., 2003; Glass et al., 2011; Wang et al., 2013). Satellite muscle cells can differentiate into osteoblasts and chondrocytes in vitro and in vivo (Abou-Khalil et al., 2015; Asakura et al., 2001; Cairns et al., 2012; Liu et al., 2011b; Morrison et al., 2006). Skeletal muscle produces osteogenic-related factors such as insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2) (Hamrick, 2011; Hamrick et al., 2010). In particular, skeletal stem cells are recruited by growth factors, including bone morphogenetic proteins (BMPs) at the fracture site, by inflammatory and bone cells (Rosen, 2011; Tsuji et al., 2006; Yu et al., 2010b) in order to contribute to bone regeneration (Bosch et al., 2000; Liu et al., 2011b; Wright et al., 2002). The involvement of BMPs, however, in the signaling pathways responsible for the muscle-bone transdifferentiation process is still unclear.

BMPs are members of the transforming growth factor β (TGF- β) superfamily. They have been shown to control osteoblast proliferation and differentiation and induce ectopic bone formation in vivo when implanted into muscle tissue (Bouyer et al., 2016; ten Dijke et al., 2000; Heldin et al., 1997). BMP-2, BMP-4 and BMP-7 are key molecules for normal bone development in vertebrates and are able to alter the C2C12 mesenchymal pluripotent cell lineage from the myogenic to the osteogenic phenotype (Akiyama et al., 1997; Crouzier et al., 2011b; Yamamoto et al., 1997). BMPs are recognized through heterodimeric complexes of transmembrane type I and type II Ser/Thr kinase receptors that then propagate signals through the Smad pathway and Smad proteins play a critical role in mediating BMP-induced signals from the cell surface to the nucleus; heterodimeric Smad complexes function as effectors of BMP signaling by regulating transcription of specific genes (Massagué and Wotton, 2000). Besides its role in bone differentiation, BMP-2 appears to control cytoskeletal rearrangement and cell migration, suggesting a role in mechanotransduction (Gamell et al., 2008; Kopf et al., 2014). We have shown that BMP-2 presented in a matrix-bound manner controls cell fate by inducing bone differentiation in vitro and in vivo (Crouzier et al., 2009, 2011a).

To achieve the correct tissue architecture during morphogenesis, cells must interact with each other and with the extracellular matrix (ECM). These interactions are mediated by two classes of adhesion receptors, cadherins and integrins, which are mechanically interconnected to drive tissue patterning and to maintain tissue integrity (Berrier and Yamada, 2007; Chen and Gumbiner, 2006; Mui et al., 2016; Weber et al., 2011). We have previously shown that BMP-2 receptors and β 3 integrins cooperate and converge to couple cell migration and fate commitment by controlling early steps of cell spreading and Smad signaling (Fourel et al., 2016). Given the change in the biomechanical properties of the ECM during the time window of muscle-osteogenic trans-differentiation, we addressed the question whether BMP-2 might operate by itself a switch in the cell adhesive machineries to guide bone tissue formation. We more particularly focused on the kinetics of adaptation of precursor C2C12 cells to BMP2 microenvironment to drive muscle or bone fate decision. Here, we provide evidence that BMP2 is sufficient to change the receptor adhesion repertoire and ECM composition specific for bone tissue. Our results show that different sets of integrins and cadherins may split the job by having somewhat complementary mechanical roles during the time window of muscle-osteogenic trans-differentiation. In bone cells, whereas β 3 integrin and β 5 integrin are working with N-cad to control transcriptional activities, integrin β 1 and cad-11 are dedicated to cell mechanics by both shaping octagonal cells and organizing the extracellular matrix.

2.2. Results

2.2.a. Matrix bound BMP-2 and soluble BMP-2 drive the muscle-bone transdifferentiation

Different laboratories, including ours, have shown that C2C12 myoblasts begin the differentiation process into bone cells after cell culture for 24 h in the presence of soluble BMP-2 (sBMP-2) on plastic, or film-bound BMP-2 (bBMP-2). bBMP-2 presentation was used to mimic in vitro the likely context of BMP-2 presentation in vivo. Indeed both sBMP-2 and bBMP-2 treatments inhibit myotube formation and induce osteoblastic differentiation, as characterized by morphological changes, alkaline phosphatase activity, phosphorylation of Smad and Osteocalcin production (Crouzier et al., 2009, 2011b; Katagiri et al., 1994a). To progress along the muscle-bone transdifferentiation, we analyzed the ability of bBMP-2 and sBMP-2 to inhibit muscle-specific transcription factors and to activate bone-specific transcription factors during the five first days of BMP-2 induction (Fig. 1A). First we verified that bBMP-2 and sBMP-2 induce the same kinetics of gene expression. After 1 day in differentiation medium without BMP-2, the expression of muscle-specific transcription factors mRNA such as MyoD and Myogenin reached a plateau at 10 and 10000 times higher, respectively, than before differentiation induction (D-1) (Fig. 1B and 1C). Whereas, there was no change in osteogenic markers such as Osterix and Osteocalcin. The increase of MyoD and Myogenin mRNA is correlated with an increase in their presence in the nucleus for 60 % of cells for MyoD after one day (D1) of differentiation and 35 % of cells for Myogenin after three days (D3) (Fig. 1B' and 1C'), suggesting the translocation of MyoD and Myogenin proteins to the nucleus in the absence of BMP-2 (Fig. 1B" and 1C"). In contrast, sBMP-2 or bBMP-2 induced an increase of mRNA specific to osteogenic differentiation; Osterix and Osteocalcin reached both a plateau at D1 that was 1 000 times higher than at D-1 (Fig. 1D and 1E). In addition, bBMP-2 films enhanced Osterix translocation to nucleus (Fig. 1D' and 1D"). Importantly, our results show that the film does not change either the kinetics of gene expression or the nuclear localization of the transcription factors as compared to plastic substrate.

bBMP-2 is able to induce mineralization as confirmed by Alizarin red staining at 3 weeks. The same results were observed on plastic with continuously renewed sBMP-2 (**Fig. 1E'**). The typical shape of calcium aggregates, observed by Scanning Electron Microscopy (SEM) (**Fig. 1E''**), confirmed the mineralization obtained after BMP-2 stimulation. In addition, the presence of calcium and phosphate in these aggregates was confirmed by Energy Dispersive X-ray analyses (Data not shown). Together our results show that both sBMP-2 and bBMP-2 are able to induce cells to switch from expressing muscle markers to expressing bone markers. Of note, bBMP-2 was sufficiently stable and bioactive to induce osteogenesis and mineralization in C2C12 cells. Finally, the bBMP-2 is as efficient as the sBMP-2 to inhibit the myogenic differentiation of C2C12 cells and induce bone differentiation.

2.2.a. bBMP-2 and sBMP-2 induce the expression, secretion and remodeling of bonespecific ECM

Next, the production of bone-like ECM was characterized in conditions in which C2C12 cells were cultured either on plastic with sBMP-2 or on films with bBMP-2. Laminin mRNA was expressed in early stages by C2C12 cells without BMP-2 stimulation whatever the substrate used (Fig. 2A). Of note, half of the increase at the plateau was reached after only one day. Immunofluorescence staining confirmed the deposition and remodeling of Laminin in thin fibers around the cells when they were cultured without BMP-2 (Fig. 2A'). In contrast, BMP-2, whatever the mode of presentation, was sufficient to inhibit the expression and deposition of Laminin, which is specific of muscle ECM, as visualized by immunostaining (Fig. 2A and 2A') or dot blot quantification (Fig. 2A''). Regarding the Fibronectin and the Collagen I, two crucial proteins in bone tissue, their gene expression largely increased with sBMP-2 and bBMP-2 (Fig. 2B and 2C). With BMP-2, Collagen I gene expression increased from D-1 and reached a plateau at D1, which was 10-times higher than at D-1. The gap between Fibronectin gene expressions with versus without BMP-2 appeared later, from D0. However, in contrast to Collagen I, Fibronectin, also a muscle-ECM protein, was already expressed in C2C12 myoblasts at D-1. Similarly to what we observed previously, the kinetics of gene expression on plastic with sBMP-2 was comparable to that on bBMP-2 films. Contrary to its gene expression, the Fibronectin deposition was observed equally with and without BMP-2 stimulation (Fig. 2B') In addition, independent of the presence of BMP-2, the Fibronectin amount was comparable at D5, either on plastic or films (Fig. 2B"). However, Fibronectin remodeling was different with or without BMP-2. Without BMP-2, cells stretched out Fibronectin in long and thin fibers, following their own aligned arrangement. In the presence of BMP-2, cells localized Fibronectin in short and thin fibers between cells, following the octagonal-shape of cells. The observed octagonal cell shape is coherent with the physiological aspect of bone cells.

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2



FIGURE 1: bBMP-2 and sBMP-2 drive the muscle-bone transdifferentiation. The ability of the (PLL/HA) films and the bBMP-2 (PLL/HA) films to induce myogenic (blue) and osteogenic (green) differentiation of C2C12 cells, respectively, were similar to classic conditions on plastic without or with 600 ng/mL of BMP-2 in solution. The scheme of the analysis is detailed in (A). C2C12 cells were seeded on films, loaded or not with BMP-2. As a control, cells were also seeded on plastic, with or without BMP-2 in solution. 1 day after seeding, set arbitrarily at D0, when cells are confluent, the medium was changed to differentiation medium. The medium was renewed at D3 and D5. The differentiation kinetics were analyzed by measuring the gene expression of MyoD and Myogenin for myogenic differentiation, and Osterix and Osteocalcin for osteogenic differentiation. In addition, the translocation of transcription factors Osterix, MyoD and Myogenin were quantified at D0, D1, D3 respectively; and the ability of films to induce mineralization was assessed at 3 weeks. The comparison of MHC and ALP activity on films versus plastic was already carried out in (Monge et al., 2012) and (Fourel et al., 2016). (B, C, D) Gene expression of MyoD, Myogenin and Osterix, quantified by RT-qPCR, on films

(dotted line) with (green) or without (blue) BMP-2 from seeding to D5 was similar to on plastic (solid line). At D0, D1 and D3 respectively, Osterix, MyoD and Myogenin were stained in immunofluorescence (B', C', D'). The nuclei are highlighted with a white circle for emphasis. The corresponding percentages of positive cells (B'', C'' and D''), i.e. with a nuclear intensity of protein above the threshold The scale bar of immunofluorescence observations is 50 μ m. (E) Gene expression of Osteocalcin on films from seeding to D5, with or without BMP-2, were similar to on plastic. (E') Alizarin red staining of calcium aggregates highlighted the conservation of the BMP-2 signal over time when BMP-2 was presented bound to the matrix. Scale bar is 400 μ m. The typical shape of calcium aggregates on BMP-2-loaded films was observed by scanning electron microscopic (SEM) (E''). Bar, 10 μ m. Ubiquitin C, GADPH and YWHAZ were used as House Keeping Genes for RT-qPCR.

Collagen I production was also induced by BMP-2 for either mode of BMP-2 presentation Dot blot quantification revealed a 3-fold increase in collagen production in presence of BMP-2, regardless of the BMP-2 presentation mode (**Fig. 2C''**). However, immunofluorescence staining revealed the presence of Collagen I only within the cells (**Fig. 2C'**). We hypothesize that either the lack of ascorbic acid prevented Collagen I secretion or the epitope was hidden, preventing antibody binding. Since the difference in the amount of Collagen I between conditions with or without BMP-2 were visible and quantifiable in dot blot, the second hypothesis is the most plausible. These results indicate that the presence of BMP-2, whether soluble or bound to the matrix, induces the production of osteogenic ECM.

2.2.b. bBMP-2 and sBMP-2 induce the expression switch towards Collagen-specific integrins and cad-11

Because of the large number of adhesion receptors, an *in silico* screening was performed using RNA sequencing data from UCSC genome browser (ENCODE data base). This website offers access to the genome sequencing of many species as well as RNA sequencing. This data base allowed us to identify which adhesion receptors are expressed the most in myotubes and in osteoblasts. Therefore, we were able to focus on the most expressed adhesion receptors. First, the distribution of each adhesion receptor, including integrins (α and β chains, respectively named ITGA and ITGB) and cadherins, in each cell type was analyzed (**Fig. 3A**). The ITGB pie charts were similar for muscle and bone cells: ITGB1 was the most expressed (78-79 %) following by ITGB5 (19 %) and ITGB3. Expressions of other ITGBs, below 1 %, are not represented in the figure. The distributions of ITGAs were more characteristic of each cell type. In decreasing order, the muscle cells expressed ITGA5 (28 %), ITGA7 (27 %), ITGA3 (20 %), ITGA6 (10 %) and ITGAV (8 %), whereas the bone cells expressed ITGA11 (43 %), ITGAV (25 %) and ITGA5 (24 %). Finally, and as expected, M-cad and N-cad were the most expressed cadherins in muscle cells (Charrasse et al., 2003),



Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2

FIGURE 2: bBMP-2 and sBMP-2 induce the expression, secretion and remodeling of bone-specific ECM. (A, B, C) Gene expression of Laminin, Fibronectin and Collagen I on films (dotted line) with (green) or without (blue) BMP-2 from seeding to D5 were similar to plastic (solid line). At D5, Laminin (A'), Fibronectin (B') and Collagen I (C') were stained by immunofluorescence and ECM proteins were quantified by dot blot (A'', B'' and C'' respectively). For a loading control, actin was also quantified. Scale bar is 150 µm. n. d. means not yet determined.

while cad-11 was the most expressed cadherin in bone cells. These pie charts allow us to identify the adhesion receptors that might be affected by BMP-2 stimulation. These pie chart also suggest a switch of adhesive receptors upon BMP-2 stimulation. To select adhesion receptors that might be the protagonists in the differentiation switch from muscle cells to bone cells, the ratios of the expression of each adhesion receptor in muscle versus bone cells were calculated (**Fig. 3B**). To identify which adhesion receptors were important at each step

of transdifferentiation of C2C12 cells, their gene expression kinetics were measured during the myo- and osteo-differentiation through qPCR and western blot analysis (**Fig. 4**). As predicted previously, ITGB expression was relatively similar in bone and muscle cells (**Fig. 4A**). However, the relative expression of ITGAs and cadherins (Fig. 4B, 4C and4D) allowed us to discriminate between the two cell types (**Fig. 3B**). Indeed, ITGA3, ITGA6 and ITGA7 genes, which encode for the 3 Laminin-specific ITGA receptors, are specific for muscle cells and were predominantly expressed in absence of BMP-2 as visualized by qPCR data (**Fig. 4B**), by western blot analysis (**Fig. 4B**') and by western blot quantification (**Fig. 4B**'').



FIGURE 3: Use of the ENCODE database to identify common and specific integrins and cadherins to muscle and bone tissue. (A) Percentage of expression of ITGB chains, ITGA chains and cadherins in muscle (top) or bone cells (bottom) were obtained by analyzing RNA sequencing data made for the ENCODE public research project. Pie charts illustrate the predominance of certain adhesion receptors in each cell type, especially for ITGA chains and cadherins. To highlight the specificity of the adhesion receptor repertoire in muscle versus bone cells, the ratio of muscle versus bone expression was calculated (B). Table (C) summarizes the highlighted adhesion receptor repertoire for each cell type. The underlined adhesion receptors were the most studied in the literature in this context of differentiation.

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2



FIGURE 4: bBMP-2 and sBMP-2 induce the expression switch towards Collagen-specific integrins and cad-11. Kinetics of gene expression of ITGB chains (A), Laminin-specific (B), Fibronectin-specific (C) and Collagen-specific (D) ITGA chains, and cadherins (E) were quantified by RT-qPCR on plastic without (blue) or with (green) BMP-2 in solution. Western Blot analysis showed the respective corresponding kinetics of protein amount (A', B', C', D' and E'). For a loading control, the membranes were incubated with an anti-actin antibody. (A'', B'', C'', D'', E'') Corresponding intensity analysis (ratio of adhesion receptor to actin) obtained by Western Blot for the different conditions. Ubiquitin C, GADPH and YWHAZ were used as House Keeping Genes for RT-qPCR. n. d. means not yet determined.

ITGA5 and ITGAV mRNA (**Fig. 4C**), which encode for the Fibronectin-specific ITGA chains, were similarly expressed with or without BMP-2 stimulation according to the western blot (**Fig. 4C', Fig. 4C''**). In contrast the ITGA1, ITGA2 and ITGA11 genes, which encode for the Collagen-specific integrins localized in bone cells, were expressed upon BMP-2 stimulation as judged by qPCR analysis (**Fig. 4D**) and by western blot (**Fig. 4D', Fig. 4D''**). BMP-2 stimulation has illustrated the switch between the muscle-specific M-cad and the bone-specific cad-11 upon BMP-2 stimulation. However, N-cad has been considered as a common cadherin, since its mRNA expression and protein amount were not affected by the presence of BMP-2. Overall our results demonstrate a switch of integrins and cadherins upon BMP-2 stimulation, thus highlighting a potential role of cad-11 and Collagen receptors to support bone differentiation.

2.2.c. BMP-2 orchestrates integrin-cadherin cross-talk to drive bone differentiation

After demonstrating a switch of integrins and cadherins upon BMP-2 stimulation, we addressed the question of whether or not a cooperation between integrin and cadherin might be involved in the bone differentiation. For this purpose, we examined the Smad response by monitoring the BMP-responsive element luciferase reporter gene (Fig. 5A) and the non-Smad response by following the expression of alkaline phosphatase (Fig. 5B) after treatment of cells with SiRNA directed against cadherins, ITGAs and ITGBs. Our results showed that the deletion of ITGB3 and N-cad leads to the most drastic decrease of luciferase signal with 60 % extinction of the signal as compared to the control and the deletion of ITGB5 and Mcad which generated a low luciferase signal decrease (22 and 37 % respectively). Of note all the depletions of ITGA3, ITGA6, ITGA7, ITGA5, ITGA1, ITGA2 and ITGA11 induced a signal decrease, from 22 to 55 %. This moderate effect can be explained by a compensatory effect between ITGAs. The depletion of ITGAV engendered a strong decrease of the luciferase signal with BMP-2 (90 %), but was also associated with a rounding up of the cells. This effect is likely due to the targeting of both $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins. Whereas the deletion of cad-11 had no effect on luciferase signal, the same deletion lead to a decrease of 80 % of ALP activity (Fig. 5B), which correlates with a decrease of ALP ARNm expression (Fig. 5B'). The effect of cad-11 silencing was quite specific since the silencing of ITGB1 and ITGB3 did not significantly induced a decrease of the ALP activity. ALP activity was decreased by 24 % with si-ITGB5, by 29 % with si-Mcad and by 47 % with si-Ncad. In

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2



FIGURE 5: BMP-2 orchestrates integrin-cadherin cross-talk to drive bone differentiation. (A) Luciferase activity of p(BRE) luciferase-transfected C2C12 cells was measured after 24 h plating on plastic with (green) or without (blue) BMP-2 in solution in the presence of various SiRNA directed against cadherins, ITGAs and ITGBs (see list in supplementary data). (B) ALP activity after 3 days of differentiation of C2C12 cells, with (in green) or without (in blue) BMP-2 in solution in the presence of various SiRNA directed against cadherins and ITGBs. (B') Corresponding relative ALP gene expression of conditions with BMP-2 measured by RT-qPCR. Elongation factor-1, GADPH and YHAZ were used as House Keeping Genes. (C) Percentage of positive cells for Osterix, for which the Osterix amount in the nucleus, detected in immunofluorescence, was 1.8-times higher than the Osterix amount detected in negative control nucleus. # means conditions with BMP-2 are not significantly different to the control without BMP-2. *p<0.005

addition, the deletion of cad-11 and ITGB5 induced a significant decrease of Osterix translocation (**Fig. 5C**), which is related to both Smad and non-Smad pathway (Celil et al., 2005). Finally, our experiments identify a coupling between ITGB3 and N-cad to regulate Smad activity whereas cad-11 would be more involved in the non-Smad pathway.

Next, we wondered if a coupling between integrins and cadherins exists to organize the bonelike ECM. To address this question, we analyzed Fibronectin deposition and remodeling in response to BMP-2 with SiRNA directed against cadherins and ITGBs. On plastic, compared to the control where Fibronectin is localized in thin fibers delimiting the octagonal-shape of cells, the deletion of ITGB1 and cad-11 lead to a disorganized Fibronectin deposition (Fig. 6A). This specific arrangement of Fibronectin was accompanied with a loss of the octagonal shape and with a disorganization of the cell layer. On plastic, this disorganization manifested itself by the presence of overlapping cell layers (data not shown). At higher magnification, long and aggregated fibers were observed with si-ITGB1 and si-cad11, compared to thin, short and numerous fibers in the control condition. Interestingly, the Fibronectin remodeling observed after depletion of ITGB1 and cad-11 was comparable to the one observed in conditions without BMP-2 (Fig. 2B'). It is worth to note that the bound presentation of BMP-2 increases the ability of cell to form bone nodules (Fig. 6C). In addition, even if the cell organization is different on films, ITGB1 and cad-11 were also crucial for tissue organization since their deletion disturbed the formation of cell aggregates. These results suggest that ITGB1 and cad-11 might be involved in mechanical coupling to drive ECM organization.

2.3. Discussion

Upon BMP-2 stimulation, the osteoblastic lineage commitment in C2C12 myoblasts is associated with a microenvironmental change that occurs over several days (Ozeki et al., 2007). This change implies adapted cell interactions with the Extracellular Matrix (ECM) and neighboring cells. Both interactions are mediated by integrins and cadherins, respectively, and are critical for bone tissue morphogenesis and architecture (Marie et al., 2014). Our findings highlight the switch of integrin and cadherin expression during muscle-bone transdifferentiation upon BMP-2 stimulation. Whereas C2C12 muscle cells express M-cad and Laminin specific integrins, the BMP-2 induced transdifferentiation into bone cells is associated with an increase in the expression of cad-11 and Collagen-specific integrins, in agreement with the literature (Di Benedetto et al., 2010; Greenbaum et al., 2012; Lai et al., 2005). Our study reveals how integrins and cadherins can work in a combinatorial fashion

Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2



FIGURE 6: BMP-2-induced Fibronectin remodeling is dependent of a ITGB1 and cad-11. (A) Microscopic observations of Fibronectin network (white). Fibronectin was stained by immunofluorescence after 3 days of differentiation of C2C12 cells on plastic with BMP-2. Bar, 600 μm. (B) Confocal microscopy

observations of Fibronectin fibrillogenesis after 3 days of differentiation of C2C12 cells on plastic with BMP-2. Fibronectin (green) was stained by immunofluorescence and nuclei were stained with Dapi (blue). Bar, 150 µm. (C) Confocal microscopic observations of cell aggregates after 3 days of differentiation of C2C12 cells seeded on bBMP-2 films. Fibronectin (green) was stained by immunofluorescence and nuclei were stained with Dapi (blue). Bar, 300µm.

and exert distinct functions to drive the osteogenic program and to control bone cell plasticity. Indeed, our results show that different sets of integrins and cadherins might have somewhat complementary mechanical roles during the time window of muscle-osteogenic transdifferentiation. In bone cells, whereas ITGB3 and ITGB5 are working with N-cad to control transcriptional activities, ITGB1 and cad-11 are both involved in ECM and tissue mechanics.

Interplay between N-cad and ITGB3/ITGB5 to regulate transcriptional activities

Our previous work demonstrated that BMPR–ITGB3 cross-talk is important for the establishment of a transient new phenotype before the conversion from myoblasts to osteoblasts (Fourel et al., 2016). In this present study, we show that at later stages of differentiation ITGB3 and ITGB5 may act in concert with N-cad to regulate Smad and non-Smad pathways (ALP) upon BMP-2 stimulation. ITGB3 is more involved in Smad signaling as already shown (Fourel et al., 2016). Our results showing the decrease of ALP and nuclear Osterix after ITGB5 deletion add another piece of evidence showing the involvement of ITGB5 in bone differentiation in a non-Smad dependent manner (Celil et al., 2005; Lai et al., 2005). ITGB5 may control osteogenesis by regulating Wnt/ β -catenin signaling through its interaction with a protein named half LIM domains protein 2 (FHL-2) (Hamidouche et al., 2009; Lai et al., 2005; Martin et al., 2002).

Controlling GSK3 activity might be an intermediate step for adhesive receptors to control osteogenic genes. Indeed, GSK3 inhibition is central to control both the intensity and the duration of Smad and non-Smad signals. We have already proposed a model wherein ITGB3 is a key element that acts at earlier steps in a multistep process by controlling both the phosphorylation of Smad1 by BMPR and the stability of pSmad1 through the repression of GSK3 activity (Fourel et al., 2016). Of note ITGB5 and GSK3 have been both identified as osteosarcoma markers (Le Guellec et al., 2013). In addition, N-cad is involved in GSK3 and β -catenin phosphorylation through Akt activation (Zhang et al., 2013) and mediate osteogenesis by regulating Osterix through PI3K signaling and GSK3 (Guntur et al., 2012). However the synergy between BMP and Wnt/ β -catenin pathway also imposes a tight regulation of GSK3 inhibition. Indeed canonical Wnt signaling is required for BMP-2-

induced bone formation (Chen et al., 2007) by participating in GSK3 activity inhibition to extend the duration of the Smad1 signal and to stabilize β -catenin (Fuentealba et al., 2007; Ikeda et al., 1998; Kishida et al., 1998; Logan and Nusse, 2004; Vinyoles et al., 2014; Zeng et al., 1997). GSK3 inhibition is important for ALP signaling which is controlled by β -catenin (Bain et al., 2003). The cooperative effects of BMP and the Wnt/ β -catenin canonical pathway are important to form cooperative transcriptional complexes to activate the osteogenic target gene Osterix (Marie et al., 2014; Rodríguez-Carballo et al., 2011). The cross-talk between adhesive receptors and BMP to control Wnt/ β -catenin pathway and drive the bone transcriptional program still need to be investigated. At first glance, FHL2 might be an interesting shuttle between ITGB5 integrin and N-cad knowing its ability to interact with both ITGB5 and β -catenin (Labalette et al., 2004; Lai et al., 2005; Martin et al., 2002). The functional link between BMP-2 and FHL2 through adhesive receptors opens new avenues in the field of osteogenesis.

Cooperation between cad-11 and ITGB1 to control ECM and tissue mechanics

Mechanotransduction, which is the transduction of mechanical forces to biochemical signals, is an important mechanism regulating both cellular and matrix mechanics to control bone maintenance and regeneration (Shih et al., 2011). In line with this, our most intriguing result was the reorganization of Fibronectin during transdifferentiation of C2C12 cells. Whereas Fibronectin fibrils were elongated by C2C12 cells before bone differentiation, Fibronectin, under short fibrils, localized at cell-cell contacts whatever the presentation of BMP-2 (Fig. 6). Our experiments also show that BMP-2 does not affect only the reorganization of Fibronectin, but also its amount of expression (Fig. 2) as already described (Fourel et al., 2016; De Jesus Perez et al., 2011). Only the deletion of ITGB1 and the deletion of cad-11 were able to rescue the stretching of Fibronectin fibrils obtained without BMP-2 treatment. Many data have shown that ITGB1 plays an important role in osteoblast differentiation and function (Hamidouche et al., 2009; Moursi et al., 1997; Wang and Kirsch, 2006; Xiao et al., 1998). Mice expressing a dominant-negative ITGB1 subunit in mature osteoblasts show reduced bone mass and defective bone formation (Moursi et al., 1997). The control of ITGB1 activation has been shown to be crucial in controlling matrix assembly and bone differentiation (Brunner et al., 2011). Consistent with this, a recent study showed that Wntinduced secreted protein-1 (WISP-1/CCN4) promotes MSC osteogenic differentiation in vitro by binding to integrin $\alpha 5\beta 1$ and enhancing the anabolic effect of BMP-2 (Ono et al.,

2011). WISP-1/CCN4 is a member of the CCN family of proteins, which are secreted ECMassociated proteins, that is highly expressed in skeletal tissues. Our present result suggests a mechanical collaboration between ITGB1 and cad-11. Even though substrate stiffness and tethering is mostly known to affect focal adhesions (Levental et al., 2009; Trappmann et al., 2012; Wen et al., 2014; Yu et al., 2010a), increasing evidence suggests that it may also affect cadherin-mediated intercellular adhesion (Ladoux et al., 2010; Smutny and Yap, 2010). It has been already shown that cad-11 and ITGB1 regulate both contractile pathway through ROCK signaling to control ECM mechanics (Alimperti et al., 2014; Brunner et al., 2011; Clark et al., 2005; Faurobert et al., 2013). The question is whether cooperation between ITGB1 and cad-11 might affect intercellular stress or the strength of adhesion to the extracellular matrix as function of bone differentiation stages. Previous studies have shown quantitatively that cells exert different traction forces on pillars covered by Fibronectin (FN pillars) depending on the type of cadherins (Jasaitis et al., 2012), and each type of cadherin might be associated with specific intercellular adhesion strengths (Chu et al., 2004, 2006). Ecadherin-mediated contact and mechanical coupling between cells are required for an increase in cell/Fibronectin traction force (Jasaitis et al., 2012). It has also been proposed that cad-11 regulates cell-cell tension necessary for calcific nodule formation by valvular myofibroblasts (Hutcheson et al., 2013). How cad-11 and ITGB1 can work together is still not understood. Their unexpected localization may provide a part of the answer. Surprisingly cad-11 has been recently localized at cell-ECM contacts in focal adhesions (Langhe et al., 2016) and fluorescence cross-correlation spectroscopy has revealed the presence of the inactive form of $\alpha 5\beta 1$ integrin at cell-cell contacts, which was under the control of N-cad in a zebrafish model (Julich et al., 2015). Altogether, these data suggest that BMP-2 might tune interplay between N-cad- and integrin-dependent signals to control cell fate by regulating the strength of adhesion to the extracellular matrix, ECM remodeling and ECM mechanics.

The role of specific integrins and cadherins expressed at different stages of osteoblast differentiation needs to be identified in order to understand signals that are modulated locally by cell adhesion molecules. Emerging evidence indicates that signaling pathways mediated by integrins and cadherins might interplay with the Wnt/ β -catenin signaling pathway to regulate osteogenic differentiation and mechanotransduction. Understanding how specific integrins or cadherins may promote osteogenic cell differentiation, bone formation, and repair may lead to novel therapeutic strategies and engineered biomaterials.
Chapitre III. Coopération entre les intégrines et les cadhérines pour contrôler la différenciation osseuse induite par la BMP-2

2.4. Materiel and methods

Buildup of Polyelectrolyte multilayer films, cross-linking and loading of rhBMP-2

Sodium hyaluronate (HA (2x10⁵ g/mol, *Lifecore Biomedical*, USA)) and poly-L-lysine (PLL (2x10⁴ g/mol, *Sigma*, France)) were dissolved in Hepes-NaCl buffer [20 mM Hepes pH 7. 4, 0. 15 M NaCl] at 0. 5 mg/mL and 1 mg/mL respectively. For all experiments, polyelectrolyte multilayer films, made of 12 bilayers of (PLL/HA), were built as previously described (Ren et al., 2008), on glass slides (VWR Scientific, France) or in 96-well plates (*Nunc*, Denmark) starting with a first layer of poly(ethyleneimine) (7x10⁴ g/mol, *Sigma*, France) at 3 mg/mL. After building, the films were cross-linked according to the protocol previously described using 1-Ethyl-3-(3-Dimethylamino- propyl) Carbodiimide (EDC, *Sigma*, France) at 70 mg/mL and N-Hydrosulfosuccinimide (sulfo-NHS, *Sigma*, France) at 11 mg/mL in [pH 5. 5, 0. 15 M NaCl] (Crouzier et al., 2009). For the bBMP-2 films, the films were pre-equilibrated in [1 mM HCl] and then loaded with BMP-2 (Clinical Grade, *Medtronic*, France) in [1 mM HCl] at 37°C for 1h30. The loaded films were thoroughly washed at least five times in Hepes-NaCl buffer in order to keep only matrix-bound BMP-2 (Crouzier et al., 2009). Finally, they were sterilized for 20 min under UV light.

Cell culture

C2C12 cells (ATCC® CRL-1772TM, < 20 passages) were maintained in polystyrene flasks in a 37°C, 5% CO₂ incubator and cultured in Growth Medium (GM [1:1 Dulbecco's Modified Eagle Medium (DMEM):F12 medium (11320-074, *Invitrogen*), 10% fetal bovine serum (FBS, 10270-098, *Invitrogen*), 10 U/mL penicillin G and 10 µg/mL streptomycin (15140-122, *Invitrogen*)]). Cells were subcultured prior to reaching 60-70% confluence. For all experiments, C2C12 cells were seeded at $3x10^4$ cells/cm² in GM until confluency (D0), when the medium was switched in Differentiation Medium (DM [1:1 DMEM:F12, 2% Horse Serum (HS, *PAA Laboratories*), 10 U/mL penicillin G and 10 µg/mL streptomycin]). For qPCR kinetics, the DM was replaced after 3 days (D3) and 5 days (D5) in DM. For conditions with BMP-2 in solution, BMP-2 was added to GM at 600 ng/mL, and refreshed at each medium change. The gene expression of the master transcription factors for myogenesis (MyoD and Myogenin) and osteogenesis (Osterix) were followed in RT-qPCR at D-1, D0, D1, D3 and D5 (**Fig. 1A**). Their translocations to the nucleus were observed by Immunofluorescence at D0 for Osterix, and at D1 and D3 for Myogenin and MyoD, respectively. In addition, the ability of bBMP-2 films to induce later osteogenesis was confirmed by following the gene expression of Osteocalcin and by verifying cell mineralization.

SiRNA interference

Cells were transfected with Small Interfering RNA (SiRNA) against β 1, β 3, β 5, α 1, α 2, α 3, α 5, α 6, α 7, α 11, α V integrins, M-, N- and 11-cadherins (ON-TARGET plus SMARTpool, Mouse, *Thermo Scientific Dharmacon*). At the same time, a scrambled siRNA (All Stars negative Control siRNA, *Qiagen*) was used as a control. The transfection was done as previously described (Fourel et al., 2016). Briefly, cells were seeded at 50 000 cells per well in a 6-well plate and cultured in GM (2 mL per well). After 15 h and 39 h respectively, GM was replaced by GM without antibiotics before adding the pre-incubated transfection mix [for one well: 6 µL of lipofectamine RNAiMAX Reagent (*Invitrogen*), 610 µL of Opti-MEM medium (*Gibco*), 1. 44 µL of 50 µM siRNA]. 24 h after the second transfection, cells were detached by trypsin-EDTA (*Gibco*) and seeded on films or plastic.

Smad assay using Luciferase reporter gene

C2C12-A5 stably transfected with an expression construct (BRE-Luc) containing a BMPresponsive element fused to the firefly luciferase reporter gene (Logeart-Avramoglou et al., 2006) were generously gifted by D. Logeart-Avramoglou (Univ Paris Diderot). Cells were cultured and transfected under the same conditions as C2C12 cells, and seeded in 96-well plates at $3x10^4$ cells/cm² in GM. After 24 h, cell lysis and luciferase measurements were carried out according to the manufacturer's instructions (Bright-GloTM luciferase assay system Luminescence, *Promega*). Measurements were normalized to the DNA content of each sample as measured by the CyQUANT assay (C7026, *Life Technology*).

Measurement of ALP activity in C2C12

At D3, cells seeded in 24-well plates were rinsed in Phosphate Buffer Saline (PBS), lysed in deionized water and stored at -80°C. Lysates were then sonicated and centrifuged at $10x10^3$ rpm for 5 min. To measure the ALP activity, 20 µL of the supernatants of lysates were added to 180 µL of [0. 1 M 2-amino-2-methyl-1-propanol (*Sigma*, St Quentin-Fallavier, France), 1 mM MgCI₂, 9 mM p-nitrophenyl phosphate (pNPP) (*Euromedex*, Mundolsheim, France), pH 10] in 96 well plates. The ALP activity was assayed by measuring the absorbance at 405 nm using a Multiskan EX plate reader (*Labsystem*, Helsinki, Finland). Total protein

amounts of each lysate were quantified using a BCA protein assay kit (*Interchim*, Montluçon, France). The activity was expressed as a percentage of the positive control activity.

Immuno-blotting and dot blot

Cells were lysed in 2X Laemmli buffer (*Sigma*). Detection of proteins by Western blotting was done according to standard protocols. ECM proteins were detected using dot blot. 1 μ L of each lysate was deposited in triplicate onto nitrocellulose membrane. Western blot and dot blot membranes were blocked at room temperature for 1 h in [10 mM Tris, pH 7. 9, 0,15 M NaCl, 0. 5% Tween 20, 5% w:w dry milk]. The membranes were incubated with primary antibodies diluted in blocking solution overnight at 4°C. After incubation with horseradish peroxidase secondary antibodies, detection of adsorbed antibodies was performed by ECL (*Amersham Biosciences, Inc.*). Normalization was done by actin detection.

Immuno-fluorescence

Cells were fixed over night at 4 °C in 3. 7% formaldehyde (FA, F1635, *Sigma-Aldrich*) in PBS. Except for ECM protein observations, cells were permeabilised for 4 min in Tris Buffer Saline (TBS) [50 mM Tris, pH 7. 4, 0. 15 M NaCl] containing 0. 2% Triton X-100. Slides were blocked in TBS containing 0. 1% BSA for 1 h, and were incubated over night at 4 °C in primary antibodies in TBS with 0. 2% gelatin. Cells were then incubated with secondary antibodies in TBS with 0. 2% gelatin. F-actin was stained with rhodamine-phalloidin and nuclei were stained using Dapi. Microscopic acquisitions were done using confocal microscope (Axiovert 200M, Zeiss, Germany) and a CoolSNAP EZ CCD camera (Ropper Scientific, Evry, France). ImageJ was used to perform all quantifications.

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from C2C12 myoblasts using a kit (*Zymo research, Proteigene,* France). Reverse transcription was done from 1 µg RNA using 5x iScript Reverse Transcription Supermix for RT-qPCR (170-8840, *Invitrogen*). Real time-quantitative PCR was performed with a thermocycler MX4800P (*Stratagène*). The reaction mix was composed of Master SYBR Green I mix (Universal SYBR Green Supermix, 172-7272, *Biorad*) - containing dNTPs, Sso7d fusion polymerase, MgCl₂, SYBR[®] Green I, ROX normalization dyes-, 0. 5 µM of each primer and 20-fold diluted cDNA. Primer efficiency was established by a standard curve using sequential dilutions of gene specific PCR fragments. According to

MIQE guidelines (Bustin et al., 2009), results were normalized to the mean of the expression levels of the 3 more stable housekeeping genes, determined with GeNorm software, and expressed as a percentage of the control condition (the trio of housekeeping genes for each experiment are indicated in each legend).

Mineralization: alizarin red staining and scanning electron microscopy (SEM) observations

C2C12 were plated at $3x10^4$ cells/cm² in GM in 24 well plate. After 2 days in growth medium, the medium was changed to mineralization medium (MM [MEM α medium (A10490-01, *Invitrogen*), 10% FBS (10270-098, *Invitrogen*), 10 U/mL penicillin G and 10 μ g/mL streptomycin (15140-122, *Invitrogen*), 50 μ g/mL ascorbic acid (A0278, *Sigma-Aldrich*) and 10 mM β -glycerophosphate (50020, *Sigma-Aldrich*)]). For conditions with BMP-2 in solution, BMP-2 was added to GM at 600 ng/mL, and refreshed at each medium change. The MM was changed every 2-3 days. Cells were maintained in culture for 3 weeks. For calcium deposition observation, cells were fixed in 3. 7% formaldehyde (FA, F1635, *Sigma-Aldrich*) in PBS for 20 min at room temperature, and the calcium was stained by Alizarin Red S staining solution [40 mM Alizarin Red S (*A5533, Sigma-Aldrich*), pH 4. 2] for 20 min at room temperature, followed by several rinsings with deionized water. Images were acquired with an Olympus BX41 microscope. For scanning electron microscopy (SEM) observations, cells were fixed with 2. 5% glutaraldehyde in 0. 1M cacodylate buffer at pH 7. 2 (C0250, *Sigma*) and dehydrated in successive alcohol baths. Cells were imaged at 2 kV using a Fei-Quanta 250 SEM-FEG.

Comparative analyses of gene expression using RNA sequencing from UCSC genome browser

RNA sequencing (RNAseq) data were exported from the Encyclopedia of the regulatory elements (ENCODE). RNAseq analysis were performed for muscle cells (7 days-differentiated C2C12 - ENCSR000AHY and primary skeletal muscle myoblast (*Homo sapiens*) - ENCSR000CWN) and bone cells (mean of responses of primary osteoblasts (*Homo sapiens*, adult 56-year female and adult 62-year male - ENCSR000CUF). Pie charts were made from the relative percentage of each of the adhesion receptors, for muscle and for bone. The adhesion receptors with a relative expression below 1 % were not represented.

Ratios were obtained by dividing the relative percentage of expression obtained in muscle to bone.

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Conclusions et Perspectives

Des communications entre l'os et le muscle existent via les facteurs de croissance et les cytokines. Ces communications inter-organes permettent le maintien de l'homéostasie tissulaire et sont critiques pendant la régénération osseuse (Glass et al., 2011; Rosen, 2011; Tsuji et al., 2006; Yu et al., 2010b). Suite à une lésion osseuse, les cellules souches dérivées du muscle sont activées durant la phase inflammatoire de la réparation (Abou-Khalil et al., 2014; Colnot et al., 2003; Glass et al., 2011). Les cellules musculaires satellites peuvent se différencier en ostéoblastes et en chondrocytes in vitro et in vivo (Abou-Khalil et al., 2015; Asakura et al., 2001; Cairns et al., 2012; Liu et al., 2011b; Morrison et al., 2006). Les muscles squelettiques produisent des facteurs liés à l'ostéogenèse comme l'insulin-like growth factor 1 (IGF1) et le fibroblast growth factor 2 (FGF2) (Hamrick, 2011; Hamrick et al., 2010). Plus particulièrement, les cellules souches squelettiques sont recrutées par les facteurs de croissance, notamment les Bone Morphogenetic Protein (BMPs) au site de lésion osseuse par l'inflammation et les cellules osseuses (Rosen, 2011; Tsuji et al., 2006; Yu et al., 2010b) pour contribuer à la régénération (Liu et al. 2011; Wright et al. 2002; Bosch et al. 2000). Le processus par lequel les BMPs sont impliquées dans les voies de signalisation responsables de la transdifférenciation MUSCLE/OS est toujours non-élucidé.

Pour obtenir une architecture tissulaire correcte durant la morphogenèse, les cellules doivent interagir les unes avec les autres et avec la matrice extracellulaire (MEC). Ces interactions sont médiées par deux classes de récepteurs d'adhésion : les intégrines et les cadhérines qui sont mécaniquement interconnectées pour conduire à la formation du tissu et au maintien de l'intégrité tissulaire (Berrier and Yamada, 2007; Chen and Gumbiner, 2006; Mui et al., 2016; Weber et al., 2011).

D'un côté, de nombreuses études rapportent une synergie entre les voies de signalisation des récepteurs d'adhésion cellule/matrice (les intégrines) et celles des facteurs de croissance (Comoglio et al., 2003; Ivaska and Heino, 2011; Margadant and Sonnenberg, 2010) sans toutefois expliquer clairement les coopérations entre les BMP-récepteurs (BMPRs) et les

intégrines. De l'autre côté, des coopérations entre les intégrines et les cadhérines ont été mises en évidence durant différents processus cellulaires (Weber et al., 2011). Ces coopérations peuvent être indirectes, *via* le cytosquelette, ou directes, la cad-11 ayant été trouvée dans les plaques focales d'adhésion (Langhe et al., 2016).

Dans ce contexte, nous nous sommes intéressés au rôle du système adhésif dans la réponse à la BMP-2 lors de la transdifférenciation des C2C12. Pour ce faire, nous avons utilisé un biomatériau, les films de (PLL/HA), afin de présenter la BMP-2 par la matrice et ainsi mimer au mieux le microenvironnement *in vivo*.

Dans une première partie, nous avons montré que, lorsque la BMP-2 est présentée par la matrice, une coopération entre les BMPRs et l'intégrine β 3 permet une réponse précoce, en modulant à la fois l'étalement cellulaire et la réponse à la BMP-2, indépendamment des propriétés mécaniques du substrat. En effet, l'intégrine β 3 permettrait la phosphorylation de SMAD par les BMPRs et la stabilité de pSmad1^{Cter} par la répression de GSK3 β , qui dégrade pSMAD.

Dans une deuxième partie, nous nous sommes intéressés à la différenciation plus tardive des C2C12, en réponse à la BMP-2. La formation et le maintien des tissus étant accompagnés d'une interaction intercellulaire, nous avons élargi notre étude du système adhésif en incluant les cadhérines. Nos résultats suggèrent une coopération entre les intégrines β 3 et β 5 et les cadhérines N et 11 dans la transdifférenciation en réponse à la BMP-2 ; β 3 et N-cad contrôleraient la voie SMAD tandis que β 5 et cad-11 interviendraient plus dans la voie non-SMAD. Enfin, nous avons vu que l'intégrine β 1 et la cad-11 sont nécessaires à l'organisation cellulaire et au remodelage de la MEC induite par la BMP-2. De plus, ces couplages ne sont pas dépendants du mode de présentation de la BMP-2, les mêmes effets ayant été observés sur les films chargés en BMP-2.

Tout comme nous avons identifié GSK3 β comme intermédiaire entre l'intégrine β 3 et la réponse à la BMP-2 à temps court, il serait intéressant d'identifier les acteurs médiés par le système adhésif dans la réponse tardive à la BMP-2. Plusieurs pistes pouvant être étudiées sont décrites ci-après.

Il a été montré que GSK3β peut aussi être inhibée par N-cad via la phosphorylation d'Akt (Zhang et al., 2013). GSK3β inhibant la voie SMAD, N-cad favorise donc indirectement la voie SMAD.

De plus, GSK3 β inhibe non seulement la voie SMAD, mais conduit aussi à la dégradation de β -caténine. Or la voie Wnt/ β -caténine permet la transcription de gènes osseux précoces, dont le gène codant pour l'ALP (Bain et al., 2003). GSK3 β est donc central dans la réponse à la BMP-2, à la fois dans la voie SMAD et dans la voie non-SMAD.

Enfin, il a été montré que la protéine Four and a half LIM domains protein 2 (FHL-2) était impliquée dans la minéralisation de la matrice et interagit directement avec l'intégrine β 5 (Lai et al., 2005). Par cette voie, l'intégrine β 5 est donc liée à l'ostéogenèse. Il serait intéressant de vérifier si, dans notre système, FHL-2 est bien l'intermédiaire entre l'intégrine β 5 et la réponse à la BMP-2, et si N-cad peut aussi réguler FHL-2.

Ensuite, nous avons vu que l'intégrine β 1 et la cad-11 participent toutes les deux à l'organisation cellulaire et au remodelage de la Fibronectine. Il est intéressant de noter qu'ici une intégrine contribue à la communication entre les cellules tandis qu'une cadhérine contribue à l'arrangement de la MEC. Cela souligne les possibles coopérations du système adhésif, et il serait intéressant d'élucider si cette coopération est directe ou indirecte via la répartition des tensions du cytosquelette. Pour déterminer si la coopération est directe, des expériences de colocalisation et de coimmunoprécipitation pourraient être envisagées. A noter cependant qu'à l'heure actuelle, aucun anticorps valable pour marquer cad-11 en immunofluorescence n'est disponible mais la construction cad-11-GFP pourrait être un bon outil. Dans le cas d'une coopération indirecte, il serait intéressant d'analyser : i) l'effet de la délétion de l'intégrine β 1 et/ou de la cad-11 sur les tensions du cytosquelette et sur les protéines de signalisation régulant la dynamique des sites d'adhésion en réponse à BMP-2 et ii) le rôle des protéines effectrices permettant de maintenir la tension du cytosquelette (ROCK, MLC) dans le réarrangement de la MEC et dans l'organisation des cellules. Enfin, une autre possibilité de coopération indirecte est envisageable : l'arrangement de la MEC serait une conséquence de l'arrangement cellulaire, ou l'inverse. En effet, nous avons vu que sans intégrine β 1, sur plastique et en présence de BMP-2, les cellules se chevauchent au lieu de former une monocouche. Or, ces zones de superpositions colocalisent avec la Fibronectine. Il serait intéressant de déterminer si la désorganisation de la Fibronectine est la cause ou la conséquence de la désorganisation cellulaire. De plus, on pourrait imaginer que ce lien de cause à effet dépend du récepteur déplété, l'intégrine β 1 ou la cad-11. L'analyse

de l'organisation cellulaire après déplétion de la Fibronectine pourrait amener des éléments de réponse. Cette analyse serait aussi intéressante pour comprendre comment les cellules forment des agrégats lorsque la BMP-2 est présentée par la matrice.

Cela nous amène à une autre question : comment la présentation de la BMP-2 affecte l'organisation cellulaire ? En effet, bien qu'aucune différence n'ait été observée en termes d'expression et de production de facteurs de transcription ostéogéniques, de protéines de la matrice ou de récepteurs d'adhésion, le mode de présentation affecte l'organisation générale du tapis cellulaire ; les cellules formant des agrégats lorsque la BMP-2 est présentée par la matrice *versus* en solution. Nous avons vu, dans la première partie, que ce mode de présentation permet une coopération entre l'intégrine β 3 et les BMPRs. L'absence d'agrégats après délétion de l'intégrine β 1 et de la cad-11 permet de supposer que, grâce à ce couplage, la cellule est sensible au mode de présentation de la BMP-2. La condensation cellulaire étant une étape cruciale lors de la formation osseuse (Hall and Miyake, 1995), cette piste de recherche permettrait, *in fine*, l'amélioration des biomatériaux.

En effet, l'étude fondamentale présentée dans cette thèse constitue une avancée dans l'amélioration des biomatériaux. De nouveaux biomatériaux sont actuellement développés ciblant spécifiquement certaines intégrines afin d'améliorer l'ostéogenèse. Par exemple, la stimulation de l'intégrine $\alpha 5\beta 1$ (Saidak et al., 2015) permet d'augmenter l'ostéogenèse, *via* la voie Wnt. De même, la voie Wnt pourrait être activée en développant des biomatériaux ciblant la N-cad (Marie et al., 2014). Nous avons vu que la présentation par la matrice de la BMP-2 permet une coopération entre l'intégrine $\beta 3$ et les BMPRs, ce qui favorise l'adhésion et la réponse précoce à la BMP-2. En déterminant les coopérations du système adhésif dans la réponse plus tardive à la BMP-2, ce biomatériau, et plus généralement les revêtements ostéoinducteurs, pourraient être optimisés par le greffage de peptides spécifiques par exemple.

Grâce à ce mode de présentation de la BMP-2, associée à une activation de récepteurs adhésifs spécifiques, il est envisageable d'obtenir une différenciation osseuse ciblée en induisant une réponse ostéogénique uniquement chez les cellules en contact avec le matériau. Ainsi, les effets secondaires indésirables liés au relargage de la BMP-2 dans la circulation, tels que l'ostéogenèse ectopique, pourraient être évités.

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Rôle et régulation des intégrines et des cadhérines dans la transdifférenciation MUSCLE/OS en réponse à la BMP-2 : approche biomimétique

Le muscle et l'os coopèrent mécaniquement mais aussi biochimiquement, via les facteurs de croissance et les cytokines. Suite à une lésion de l'os, les cellules souches sont recrutées et induites en différenciation osseuse grâce à la sécrétion de molécules bioactives telles que les facteurs de croissance. L'une des stratégies de l'ingénierie tissulaire de l'os est de combiner des matériaux avec des facteurs de croissance osseux. Les protéines morphogéniques osseuses (ou BMPs), pouvant être présentées aux cellules en solution ou enchâssées dans la matrice, appartiennent à la famille des facteurs de croissance basiques et jouent un rôle très important dans la formation de l'os. Les BMPs induisent non seulement une différenciation osseuse de progéniteurs osseux, mais induisent aussi la transdifférenciation de progéniteurs musculaires vers un phénotype ostéoblastique. L'obtention de la complexité de l'architecture tissulaire osseuse nécessite des interactions continues entre la cellule et son microenvironnement. Ces interactions sont médiées par les récepteurs cellule/matrice (intégrine) et cellule/cellule (cadhérines). Dans cette thèse, nous nous sommes intéressés au rôle du système adhésif dans la réponse à la BMP-2 lors de la transdifférenciation des myoblastes C2C12. Nous avons utilisé un film multicouche à base de hyaluronane et de poly(L-lysine) comme biomatériau pour présenter la BMP-2 par la matrice. A court terme, nous avons mis en évidence une coopération entre l'intégrine ß3 et les récepteurs BMP dans l'induction d'un étalement cellulaire et d'une réponse précoce à la BMP-2, via la protéine GSK3ß. A plus long terme, nous avons montré un switch du répertoire adhésif en réponse à la BMP-2. Enfin, nos résultats suggèrent une coopération entre les intégrines β 3 et β 5 et les cadhérines N et 11 dans la transdifférenciation induite par la BMP-2.

Muscle and bone cooperate both mechanically and biochemically, through growth factors and cytokines. Following a bone lesion, stem cells are recruited and their differentiation is induced via the secretion of bioactive molecules such as growth factors. One strategy in bone tissue engineering is to combine materials with bone growth factors. Bone Morphogenetic Proteins (BMPs), which can be presented to the cell either in solution or bound to the matrix, belong to the basic growth factor family and play a very important role in bone formation. BMPs induce not only the differentiation of bone progenitors, but also the transdifferentiation of muscle progenitors towards an osteoblastic phenotype. Obtaining the complexity of the bone tissue architecture requires continuous interactions between the cell and its microenvironment. These interactions are mediated by cell/matrix and cell/cell receptors (integrins and cadherins, respectively). In this thesis, we investigated the role of the adhesion system in the context of its response to BMP-2 during the transdifferentiation of C2C12 murine myoblasts. To do so, we used polelectrolyte multilayer films composed of hyaluronan and poly(L-lysine) as a biomaterial to present BMP-2 in a matrix-bound manner. Short term, we revealed a cooperation between the integrin β 3 and BMP receptors in the induction of cell spreading and of an early response to BMP-2 via the protein GSK3 β . In a longer term, we showed a switch in the repertoire of adhesion receptors in response to BMP-2. Finally, our results suggest a cooperation between β 3 and β 5 integrins and cadherins N and 11 for the BMP-2-induced transdifferentiation.