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Micromotifs de BMP-2 et de fibronectine sur matériaux mous: un outil pour recréer la niche de cellules souches in vitro

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Micropatterns of BMP-2 and fibronectin on soft materials: a tool for recreating the stem cell niche in vitro

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INTRODUCTION

I. Stem cell niche

1. Generalities of the stem cell niche

In vivo, adult stem cells play a crucial role in the maintenance of tissue homeostasis, as well as during tissue repair. These cells are characterized by an intrinsic potential in terms of proliferation and differentiation, which allows them to transform from an undifferentiated state capable of self-renewal to mature cells with precise tissue-dependent functions. Within a tissue, the balance between self-renewal and differentiation is crucial, as an imbalance toward differentiation would deplete stem cell populations, while excessive self-renewal can lead to an uncontrolled expansion of cells in which mutations could arise and induce tumorigenesis (D. L. Jones & Fuller, 2013).

This balance is ensured by mechanisms like asymmetric versus symmetric division (Fuchs, Tumbar, & Guasch, 2004; F M Watt & Hogan, 2000). Symmetric division occurs when a stem cell divides into two identical daughter cells, both of which maintain their ability for self-renewal.

Asymmetric division, on the other hand, occurs when the division of a stem cell results in the formation of two distinct populations, one which retains its capacity for self-renewal, and the differentiated progeny. There are two manners by which this asymmetry can be achieved, namely invariant and population asymmetry, as shown in Figure 1.

Invariant asymmetry is the mechanism by which the division of a single stem cell results in one daughter cell which remains in the niche as a stem cell, while the other daughter cell differentiates and leaves the niche. There are many examples of this phenomenon in unicellular organisms and invertebrates (F M Watt & Hogan, 2000). It should be noted that this occurs despite both daughter cells residing in the same tissue, which suggests that it is not systemic signals, but rather the integration of cues from the local microenvironment of the individual stem cell that allows the cell to regulate its fate.

Population asymmetry, on the other hand, is a much more complex regulatory mechanism, which is involved in the modulation of self-renewal and differentiation in most mammalian tissues, like the human epidermis (Jensen, Lowell, & Watt, 1999). On average, a single cell will produce one stem and one committed daughter, but unlike invariant asymmetry, population asymmetry relies on the probabilistic differentiation of stem cells within the niche, rather than individual and deterministic cell behaviors.

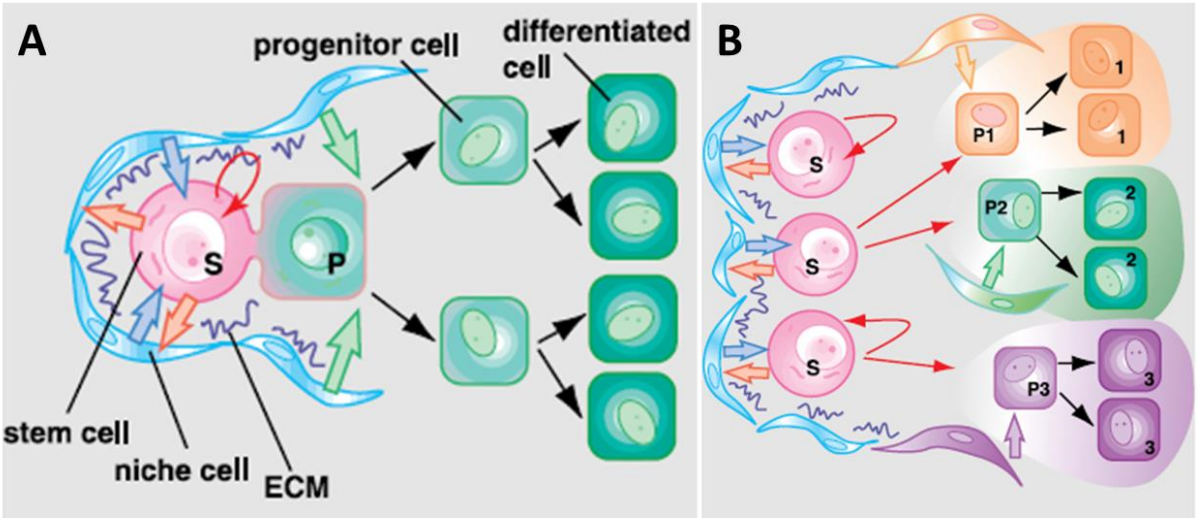


Figure 1: ***Alternative models for stem cell deployment****. (A) Invariant asymmetry. A stem cell (S) gives rise by asymmetric division to a progenitor (P) with a more restricted proliferation potential, which differentiates in response to extrinsic cues. The stem cell phenotype is regulated by reciprocal short- and/or long-range signaling (thick colored arrows). (B) Populational asymmetry. Stem cells give rise to daughter cells that can be either stem cells or progenitors that differentiate along different pathways (1, 2, and 3) depending on the combination of extrinsic factors to which they are exposed. ECM, extracellular matrix. Adapted from Watt and Hogen, 2000.*

Although very different, both types of asymmetric division are the manifestation of a shift from proliferation to differentiation. This shift is dictated by cues provided by the cell's microenvironment, which it detects through receptors at the cell membrane, and to which it responds through various signaling pathways.

This microenvironment is called the stem cell niche. The concept of niche, first established by Schofield in 1978 for hematopoietic stem cells (HSCs) (Schofield, 1978), has since been developed (Iwasaki & Suda, 2010; D. Park, Sykes, & Scadden, 2012; Sugiyama & Nagasawa, 2012). Stem cells niches have also been described for many other stem cell types, including epithelial (Tumbar et al., 2004), intestinal (Barker, 2014; Sailaja, He, & Li, 2016), follicle (Rompolas & Greco, 2014), or neural (Conover & Notti, 2008; Decimo, Bifari, Krampera, &

Fumagalli, 2012) stem cells. Within a tissue, stem cells therefore reside in certain compartments, which provide them with various signals, both biochemical and mechanical. These cues regulate and impact the intrinsic autoregulation of stem cells, and influence not only the self-renewal of the mother cell, but also the fate of daughter cells, differentiated or not.

This extremely localized signaling involved in the control of the proliferation and differentiation of stem cells is the cornerstone of the concept of stem cell niche.

2. Factors regulating stem cells in their niche

There are both intrinsic and external factors which regulate the fate of stem cells in this particular microenvironment. In the experiments described below, we modulated external parameters of the cell's microenvironment to influence its behavior. Due to the nature of our works, we will mainly be focusing, in the introduction of this thesis, on the external factors modulating stem cell behavior, rather than intrinsic factors regulating their self-renewal.

a. Intrinsic factors

Nevertheless, it is important to keep in mind that intrinsic factors exist and can influence stem cells over time. Briefly, intrinsic controls of stem cell behavior include intracellular determinants of asymmetric cell division (Bardin, Le Borgne, & Schweisguth, 2004; Y. N. Jan & Jan, 1998; Knoblich, Jan, & Jan, 1995), changes in gene expression (Cross & Enver, 1997; Korinek et al., 1998; Van Genderen et al., 1994), and internal clocks (Dugas, Ibrahim, & Barres, 2007; Kondo & Raff, 2000; Raff, 2007; Roese-Koerner, Stappert, & Brüstle, 2017; Temple & Raff, 1986; Zagni et al., 2014), which control division and stem cell aging.

This can be relevant in cell culture, during which cells are maintained and used at different levels of passage. Indeed, cell aging can change their response to the local environment. This is why, in the experiments described below, we used cells at levels of passage which were low, and similar between experiments, to ensure that the results we were observing were

indeed due to changes in the local environment, and not to changes in the intrinsic signaling of the stem cell.

These intrinsic components of stem cells are modulated by external factors. It is these factors which, collectively, make up the microenvironment, and therefore define the stem cell niche.

b. External factors

These external signals are comprised of cell-cell interactions, secreted factors and the extracellular matrix, as depicted in Figure 2.

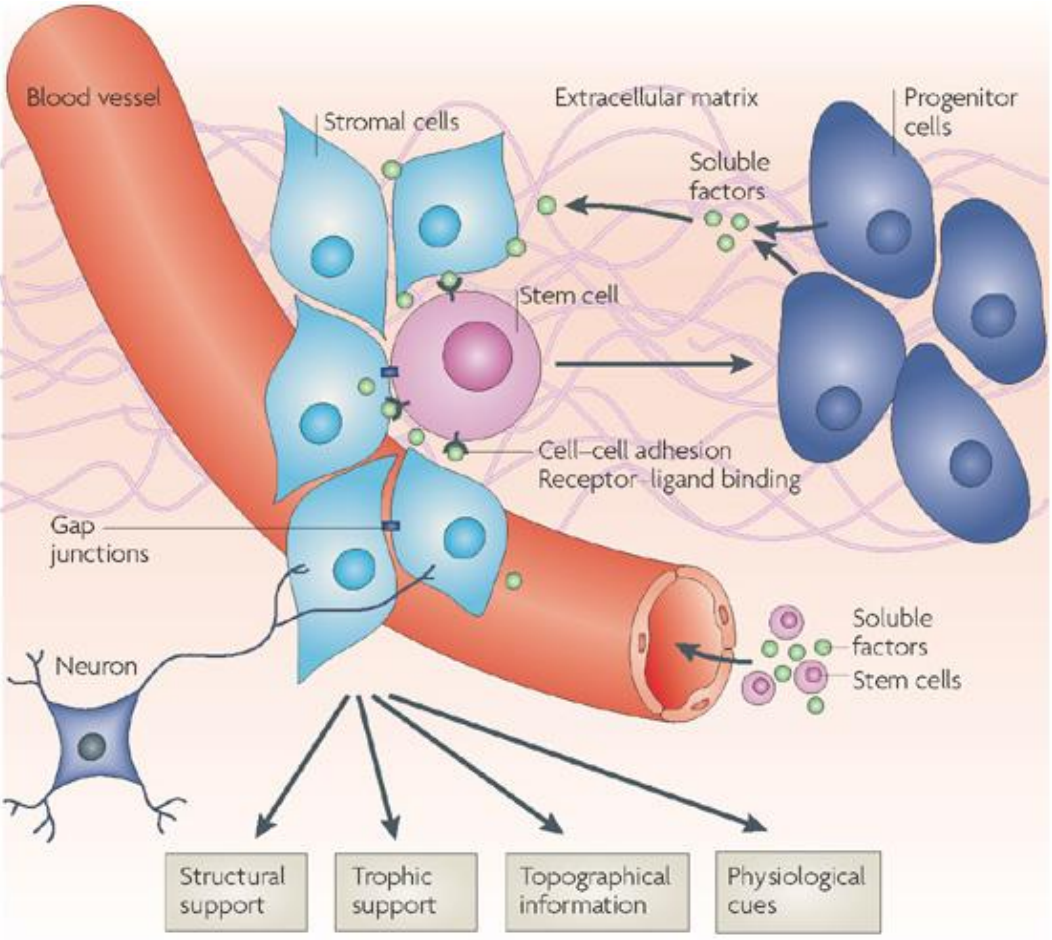


Figure 2: ***The niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators****. This schematic depicts known components of a hypothetical niche: the stem cell itself, stromal cells, soluble factors, extracellular matrix, neural inputs, vascular network, and cell adhesion components. It is important to note that although many niche components are conserved, it is unlikely that every niche necessarily includes all of the components listed. Instead, niches are likely to incorporate a selection of these possible avenues for communication, specifically adapted to the particular functions of that niche,*

which might be to provide structural support, trophic support, topographical information and/or physiological cues. From Jones and Wagers 2008.

i. Cell-cell interactions

While stem cells in their niches can be affected by their local environment at the single cell level, as evidenced by invariant asymmetric differentiation, interactions between cells have also been shown to affect stem cell fate.

Indeed, it has been demonstrated that these interactions are mediated by membrane proteins like cadherins, whose loss within germline stem cells of Drosophila disrupted adherens junctions and therefore cell-cell interactions. This was shown to result in a loss of stem cells in the fly ovary or testis (X. Song et al., 2002; X. Song & Xie, 2002).

Similarly, the transmembrane protein Notch and its ligand Delta were shown to affect differentiation of the sensory organ precursor cell in Drosophila (Artavanis-Tsakonas, Rand, & Lake, 1999). Notch signaling is also important in vertebrates, whether in the retinal neuroepithelium, the skeletal muscle, or even blood (Austin, Feldman, Ida, & Cepko, 1995; Kopan, Nye, & Weintraub, 1994; Shawber et al., 1996; Varnum-Finney et al., 1998).

Finally, cell-cell interactions were demonstrated in the HSC niche, in which direct interactions occurred between osteoblasts and HSCs (J. Y. Wu, Scadden, & Kronenberg, 2009; Jiwang Zhang et al., 2003).

ii. Secreted factors

There exists a wide range of secreted factors which play roles in the regulation of stem cell proliferation and differentiation. These factors can diffuse throughout the niche, or have a paracrine mode of action. While most of these factors are secreted proteins, a role for small molecules and ions has also been demonstrated. Examples of this are the effect of higher concentrations of calcium ions on HSC localization near osteoblasts in bone marrow (G. B. Adams et al., 2006), or oxidative stress on HSC self-renewal (K. Ito et al., 2004).

In terms of protein regulation of the stem cell niche, two families of proteins, namely Transforming Growth Factor β (TGFβs) and Wnts, play remarkably similar and crucial roles in the stem cell niches of various tissues and species. Indeed, the importance of TGFβs has been shown in the regulation of melanocyte, hair follicle, neural, epithelial, mammary, mesenchymal or embryonic stem cells (James, Levine, Besser, & Hemmati-Brivanlou, 2005; Jian et al., 2006; Kandasamy et al., 2014; Kordon et al., 1995; Moses & Barcellos-Hoff, 2011; Nishimura et al., 2010; Oshimori & Fuchs, 2012; Pineda et al., 2013). Likewise, it has been demonstrated that Wnt signaling is involved in many stem cell types, including mammary, intestinal, mesenchymal or embryonic (Baksh, Boland, & Tuan, 2007; Boland, Perkins, Hall, & Tuan, 2004; S. Liu, Dontu, & Wicha, 2005; Sumi, Tsuneyoshi, Nakatsuji, & Suemori, 2008; van Es et al., 2005).

In the context of this thesis, we will be focusing on a growth factor which is part of the TGFβ superfamily, namely an osteoinductive protein called Bone Morphogenetic Protein 2 (BMP-2), whose structure and function will be discussed in more detail below.

iii. Extracellular matrix

Finally, the extracellular matrix (ECM) plays a crucial role in stem cell regulation in the niche, through its effect on cell adhesion, but also as a reservoir for growth factors and a provider of mechanical signals.

The ECM is the noncellular component of tissues and organs. Although ubiquitously present, its composition and structure varies greatly from tissue to tissue. One of the roles of the ECM is structural, as it provides a scaffold in which the cells can function. It is also a key regulator and initiator of certain cell and tissue behaviors, such as morphogenesis, differentiation or homeostasis (Frantz, Stewart, & Weaver, 2010). Naturally, perturbations of the composition or structure of the ECM can result in a wide range of pathologies, which can arise from genetic abnormalities (Järveläinen, 2009) or environmental factors, such as the vitamin C deficiency responsible for scurvy (Olmedo, Yiannias, Windgassen, & Gornet, 2006).

Briefly, the ECM is composed of two main types of macromolecules, namely proteoglycans and fibrous proteins, as depicted in Figure 3 (Järveläinen, 2009; Schaefer & Schaefer, 2010). Proteoglycans form hydrated gels within the tissue, and serve many different functions, in particular in terms of hydration, buffering and force resistance. The fibrous proteins include collagens, fibronectin, tenascin and laminins, and serve to organize and structure the ECM. The various functions, compositions and properties of the ECM have been extensively described in the literature, and are reviewed in detail elsewhere (Frantz et al., 2010). Due to the relevance of fibronectin in the context of this thesis, its structure will be described in more detail below.

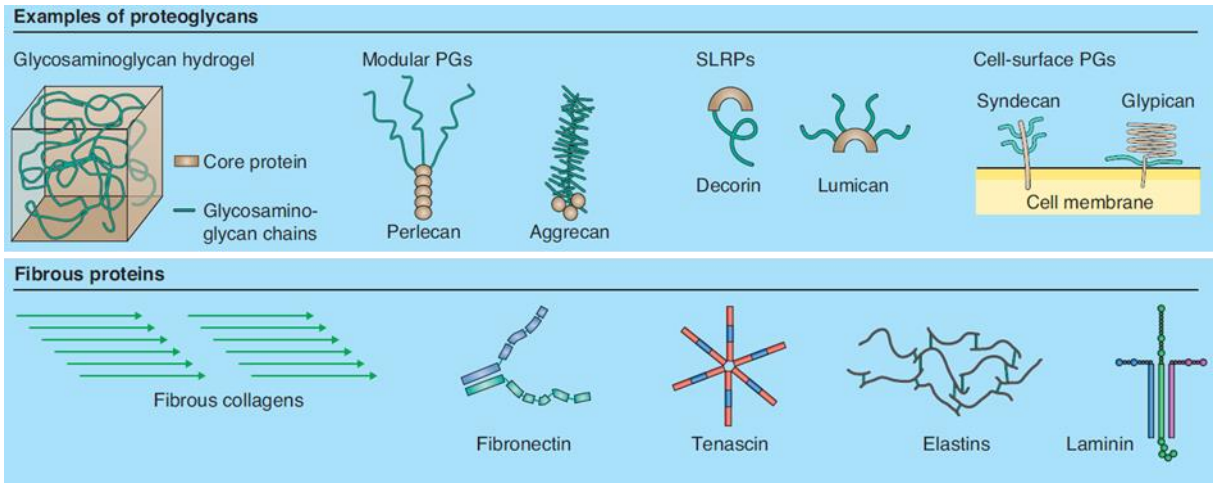


*Figure 3: **ECM macromolecules**. Abbreviations: PG, proteoglycan; SLRP, small leucine-rich proteoglycan. Adapted from Frantz et al. 2010.*

The various cues provided by the ECM are functionally integrated by stem cells, which allows them to maintain their homeostasis (Discher, Mooney, & Zandstra, 2009; Peerani & Zandstra, 2010; Pera & Tam, 2010; Fiona M Watt & Fujiwara, 2011), as shown in Figure 4.

The importance of ECM in stem cell regulation is evidenced by in vivo studies, which show that endogenous regeneration or stem cell therapies are incompatible with aged or altered ECMs (Kurtz & Oh, 2012). Further evidence of this is provided by the use of decellularized tissues, like a rhesus monkey kidney, in which the preserved ECM served as a three-dimensional scaffold guiding the differentiation of stem cells into tissue-appropriate cell types (Nakayama, Batchelder, Lee, & Tarantal, 2010).

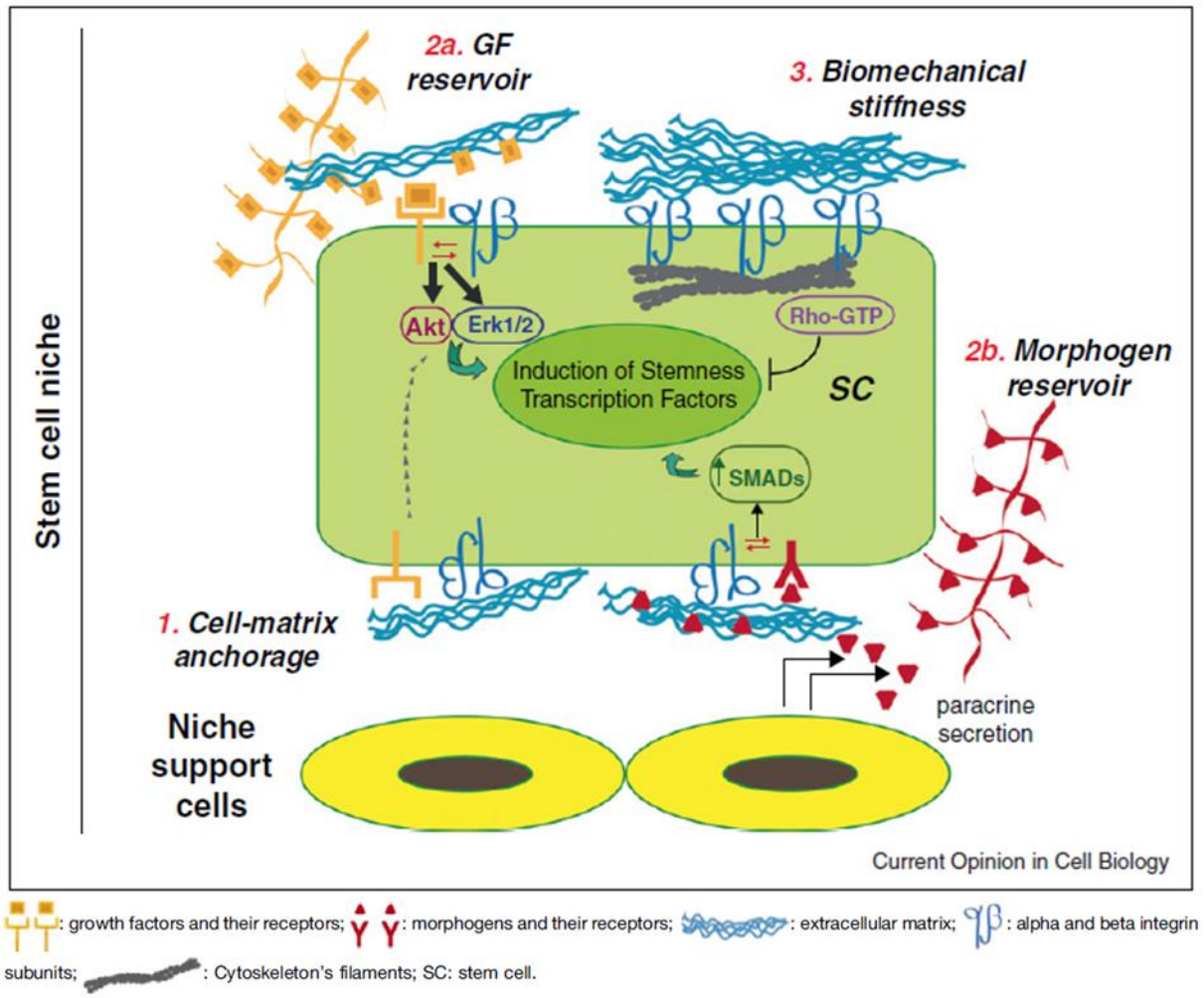


Figure 4: ***Hypothetical model of stem cell regulation by the ECM****. ECM/integrin interaction within the stem cell niche contributes to three main functions: (1) Cell-matrix anchorage, in which integrin-mediated cell adhesion physically anchors the stem cell to ECM proteins. Through these mechanisms, growth factor receptors can be activated by an integrin-dependent mechanism as well. (2) Reservoir for growth factors and morphogens: ECM binding of soluble growth factors and morphogens spatially and temporally controls their interactions with transmembrane receptors. Integrin crosstalk with other receptors regulates signaling (Erk1/2, Akt, SMADs) preserving stemness. (3) Biomechanical stiffness. ECM/integrin interaction senses mechanical forces, leading to cytoskeleton re-organization and stem cell homeostasis. From Brizzi et al. 2012*

1. Cell Adhesion

Cells are physically attached to the ECM through the intervention of membrane receptors named integrins. Integrins are important in the regulation of stem cell behavior, and anchor cells within their niche, close to the signals required for their self-renewal.

Integrins are transmembrane receptors composed of an α and a β subunit, whose extracellular domain interacts with the ECM, and who communicate with the intracellular

compartment through their cytoplasmic domain. Within adult mammalian tissues, integrin expression has been shown to be increased in stem cells.

Indeed, basal keratinocytes in the epidermis, as well as spermatogonial stem cells in mouse testis, express high levels of α6 integrin (Shinohara, Avarbock, & Brinster, 1999; Shinohara, Orwig, Avarbock, & Brinster, 2000; Fiona M. Watt, 2002). Similarly, the stem cells of multiple tissues display increased levels of β1 integrin. This was demonstrated in the interfollicular epidermis, but also in stem cells within the follicular bulge region of the outer root sheath, HSCs, and the satellite cell niche in the muscle (Brakebusch et al., 2000; Sherwood et al., 2004; Wagers, Allsopp, & Weissman, 2002; Fiona M. Watt, 2002). In the ovary of Drosophila, integrins were shown to be essential for the anchoring, proliferation, and differentiation of follicle stem cells. Interestingly, the same study showed that these cells also participate in niche maintenance by producing their own integrin ligand, namely laminin A, thus providing evidence for reciprocal communication between ECM and cells within the niche (O'Reilly, Lee, & Simon, 2008).

While these studies demonstrate the implication of integrins in stem cell maintenance, the exact role they play appears to be tissue-dependent:

- In mice, α6β1 integrin is essential for neural stem cell (NSC) adhesion to the vascular niche (Q. Shen et al., 2008), but also spermatogonial stem cell homing to the germline niche (Kanatsu-Shinohara et al., 2008).

- The α9 integrin chain binds to an ECM protein named tenascin-C (Yokosaki et al., 1994), and is involved in both the HSC and NSC niches. In vitro, murine HSCs were shown to proliferate in an integrin α9-dependent manner, and yet in vivo mice lacking Tenascin-C were shown to maintain normal hematopoiesis (Nakamura-Ishizu et al., 2012). Likewise, Tenascin-C was shown to have a crucial effect on embryonic NSCs (Garcion, Halilagic, Faissner, & Ffrench-Constant, 2004), yet its deficiency in adult mice did not affect the function of the NSC niche (Kazanis, Belhadi, Faissner, & Ffrench-Constant, 2007).

- In mouse keratinocytes, deleting the β1 integrin gene severely affected the proliferation of hair matrix cells and basal keratinocytes (Brakebusch et al., 2000). On the other hand, deleting the genes for β1 and β7 integrins in the hematopoietic system of mice did not result in abnormal hematopoiesis (Bungartz et al., 2006).

Overall, these data highlight that the role of cell surface receptors, and in particular integrin-mediated cell-ECM interactions, is not straightforward, and that there likely exist redundancies within the stem cell niche which allow the loss of one integrin to be compensated in order to maintain normal tissue function.

Although integrins are the most studied cell receptors in the context of the stem cell niches, it should be noted that other receptors have been shown to directly impact stem cell behavior. In HSCs, studies have identified other receptors which mediate stem cell retention in the niche, including the c-kit ligand steel factor (SLF), Rho guanosine triphosphatases Rac1 and Rac2, or CXC chemokine ligand 12 receptor CXCR4 (Fleming, Alpern, Uchida, Ikuta, & Weissman, 1993; Gu, 2003; Sugiyama, Kohara, Noda, & Nagasawa, 2006). Likewise, the homing of HSCs to the bone marrow during transplantation has been shown to be mediated by the adhesion receptor CD44 (Avigdor et al., 2004) and Robo4, a receptor known to be involved in axon guidance (Smith-Berdan et al., 2011).

While the implication of integrins in cell adhesion is well-documented in the literature, there exists a complementary role for integrin expression, namely the regulation of membrane receptors and signaling pathways. Indeed, integrins have been shown to regulate the cellular response to growth factors and cytokines like IL-3 or TGF-β (Defilippi et al., 2005; Ivaska & Heino, 2011; Legate, Wickström, Fässler, Fa, & Wickstro, 2009; Margadant & Sonnenberg, 2010; Uberti, Dentelli, Rosso, Defilippi, & Brizzi, 2010). Likewise, integrin expression regulates signaling pathways (Brizzi, Tarone, & Defilippi, 2012), as demonstrated in NSCs and mammary stem cells through Notch and epithelial growth factor (EGF) receptor activity (Brisken & Duss, 2007; Campos, Decker, Taylor, & Skarnes, 2006), and in intestinal stem cells, where β1 integrin deletion led to impaired Hedgehog signaling, and thus increased epithelial proliferation (R. G. Jones et al., 2006).

Overall, the works described above highlight an important role of cell adhesion receptors like integrins in stem cell maintenance within the niche, with differences between tissues suggesting that the mechanisms by which these molecules impact the stem cell behavior may be linked to a specific ECM environment and tissue-dependent functions.

2. GF reservoir

In addition to the integrin-mediated regulation of stem cell activity within the niche, ECM also plays an important role in the stem cell niche through its ability to present growth factors in a non-canonical manner. As mentioned above, local control is a crucial aspect of the niche concept, and when it comes to growth factors this is made possible through their binding to several structural elements of the ECM. This characteristic has been shown to regulate their local availability, as well as allowing a biochemical gradient (Hynes, 2009).

There are two strategies by which the ECM can present growth factors to the surrounding cells. The first involves making the growth factors insoluble, unavailable, or biologically inactive through their binding to the ECM. This has been shown to be the case for fibroblast growth factor (FGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1), as well as bone morphogenetic proteins (BMPs) and TGF-β (X. Wang, Harris, Bayston, & Ashe, 2008; Y. Zhu et al., 1999). These proteins have been shown to interact with fibronectin, vitronectin, collagens and proteoglycans, which regulate their presentation.

The second strategy for growth factor presentation by the ECM involves the action of enzymes like metalloproteinases, which degrade the structural components of the ECM. This action results in ECM remodeling, and allows the release of growth factors trapped within in an insoluble state.

Examples of non-canonical growth factor presentation have been described in the regulation of the NSC and muscle niches. For NSCs, heparin sulfate proteoglycans, part of the neural fractone ECM, were found to capture FGF-2 from the medium, and thus aid NSC function by

promoting growth factor activity in this niche (Douet, Kerever, Arikawa-Hirasawa, & Mercier, 2013; Kerever et al., 2007). For muscle satellite cells, proteoglycans in the basal lamina were shown to bind many growth factors which stimulate satellite cell survival, activation and proliferation, including FGF, HGF, EGF, IGF-1, as well as the Wnt family of glycoprotein ligands (Brack, Conboy, Conboy, Shen, & Rando, 2008; DiMario, Buffinger, Yamada, & Strohman, 1989; Golding, Calderbank, Partridge, & Beauchamp, 2007; Le Grand, Jones, Seale, Scimè, & Rudnicki, 2009; Machida & Booth, 2004; Tatsumi, Anderson, Nevoret, Halevy, & Allen, 1998). This binding to the ECM or to the satellite cell surface provides cells with a local reservoir of signaling molecules, which can be sequestered in an inactive or active form (Cornelison, Filla, Stanley, Rapraeger, & Olwin, 2001; Jenniskens, Veerkamp, & Van Kuppevelt, 2006; Langsdorf, Do, Kusche-Gullberg, Emerson, & Ai, 2007; Olwin & Rapraeger, 1992; Tatsumi et al., 1998).

We have previously mentioned the regulation of membrane receptors and signaling pathways by integrins. The interactions between ECM proteins and growth factors described here may be a strategy by which the stem cell niche potentiates cell responsiveness to growth factors through integrin-growth factor receptor crosstalk (Rahman et al., 2005; Wijelath et al., 2006), in a confined spatial context.

3. Mechanical and geometrical inputs

Another regulator of stem cell behavior is provided by the biophysical properties of the ECM. These properties, such as stiffness, are defined by the ECM components and their organization (DuFort, Paszek, & Weaver, 2011; T. Mammoto & Ingber, 2010). This explains why ECM composition varies in a tissue-dependent manner (Votteler, Kluger, Walles, & Schenke-Layland, 2010; Fiona M Watt & Fujiwara, 2011), and also why aging, disease or injury can alter the biophysical properties of the ECM (Engler, Griffin, et al., 2004; Y. Gao & Kostrominova, 2008; Rosant, Nagel, & Pérot, 2007; Stedman et al., 1991).

Within their niche, stem cells are subjected to external forces such as the compression exerted by neighboring cells, or the stiffness of the matrix. The response of cells to these forces is not passive, but rather reciprocal, as cells modify their cytoskeleton to generate internal forces, which are then in turn transmitted to the surrounding ECM through focal adhesions (Halder, Dupont, & Piccolo, 2012).

Focal adhesions are complexes which are present at the cell membrane, and composed of integrins, adaptors and signaling proteins. The role of these focal adhesions is to physically link the cytoskeleton with the ECM. As such, focal adhesions are the first link of the mechanotransduction chain which connects the ECM to chromatin, through the cytoskeleton, nuclear membrane and nuclear matrix. In the same way that the cell responds to the biophysical properties of the ECM, this chain is not a passive structure, but rather a dynamic and complex machinery that determines cell behavior in response to forces generated in the ECM (DuFort et al., 2011; Sarangi et al., 2017).

Some insight into the mechanisms of this machinery is provided below, but briefly, the bidirectional and dynamic transmission of forces between a single cell and its surrounding ECM allows the cell to maintain its shape and regulate its behavior, through isometric tension within the cytoskeleton (Halder et al., 2012; A. Mammoto, Mammoto, & Ingber, 2012; T. Mammoto & Ingber, 2010).

At the molecular level, there are many signaling pathways which have been identified in mechanotransduction, including Ras/MAPK, PI3K/Akt, RhoA/ROCK, YAP/TAZ, Wnt/β-catenin, or TGF-β pathways (Dupont, 2016; Halder et al., 2012; Sun, Chen, & Fu, 2012), as schematized in Figure 5.

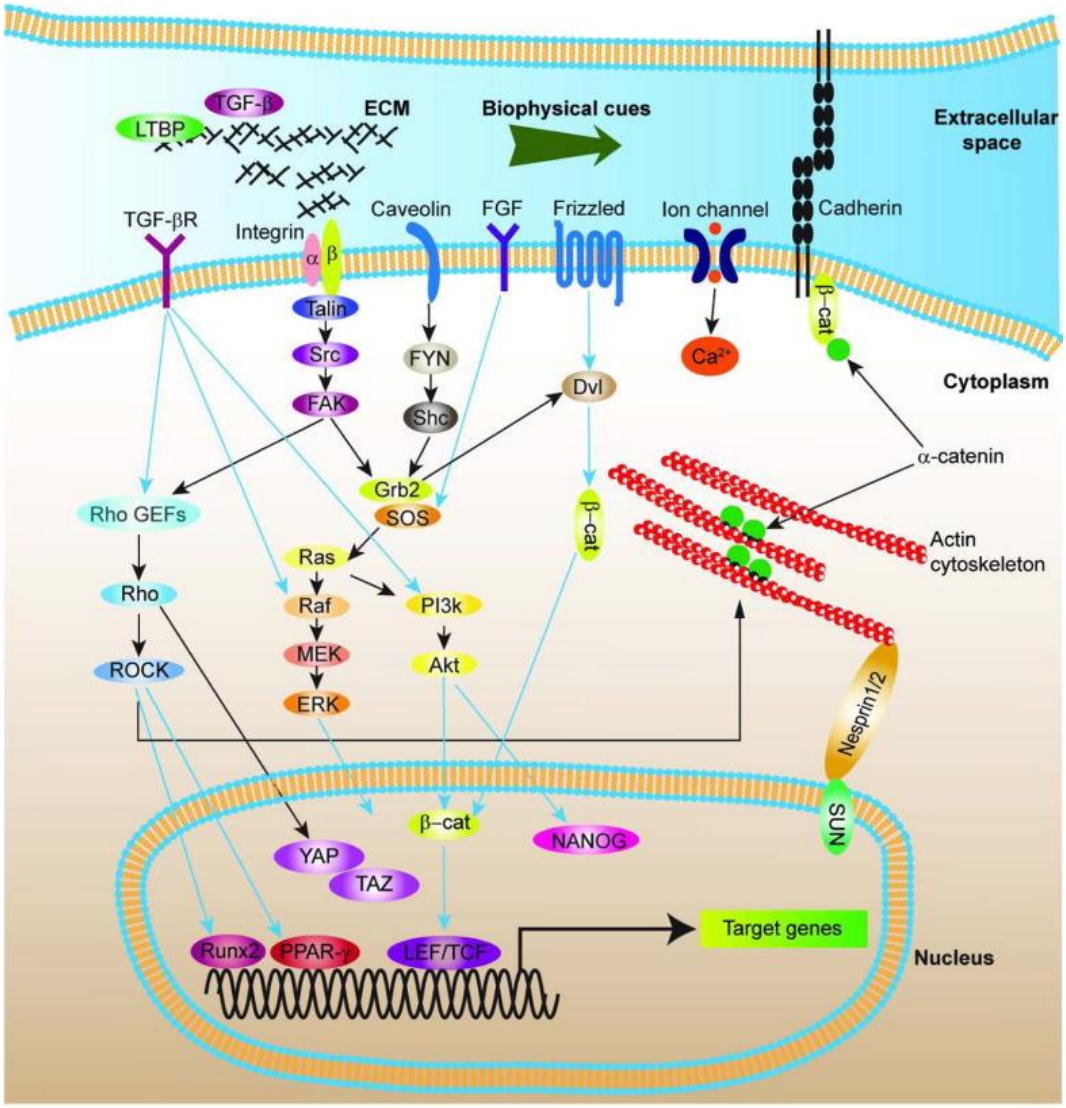


Figure 5: ***Signaling crosstalk*** *between the mechanotransductive processes (black arrows) and other known soluble factor-mediated signaling pathways regulating the fate decisions of stem cells (blue arrows). From Sun, Chen and Fu 2012.*

The effect of matrix stiffness on stem cell fate determination has been the object of several studies, both in vivo and in vitro.

In vivo, the decrease of muscle stiffness in collagen VI-null mice muscle induced reduced stem cell self-renewal in the satellite cell niche, which was rescued by the grafting of wild-type fibroblasts (Urciuolo et al., 2013).

This role of substrate stiffness in satellite cell maintenance is also supported by in vitro studies (PM Gilbert, KL Havenstrite, KEG Magnusson, A Sacco, NA Leonardi, P Kraft, NK Nguyen, S Thrun, MP Lutolf, 2011; Urciuolo et al., 2013). Similar observations have highlighted the importance of ECM stiffness in the central nervous system and the NSC niche (Georges, Miller, Meaney, Sawyer, & Janmey, 2006; Keung, De Juan-Pardo, Schaffer, & Kumar, 2011; Saha et al., 2008). In human MSCs, the elastic modulus of the underlying substrate was shown to strongly and irreversibly guide stem cell commitment toward neurogenic, myogenic or osteogenic lineages (Engler, Sen, Sweeney, & Discher, 2006). It was also demonstrated that matrix stiffness impacts human MSC self-renewal and maintenance (Winer, Janmey, McCormick, & Funaki, 2009), as well as the osteogenic differentiation of rat MSCs (P. Y. Wang, Tsai, & Voelcker, 2012). A recent review looks precisely at matrix mechanosensing and the resulting mechanisms in the cell, in particular relating to cytoskeletal forces and nuclear mechanical properties (F. Jiang & Doudna, 2017).

Examples of the influence of the mechanical properties of the ECM on stem cell fate determination and behavior are represented in Figure 6.

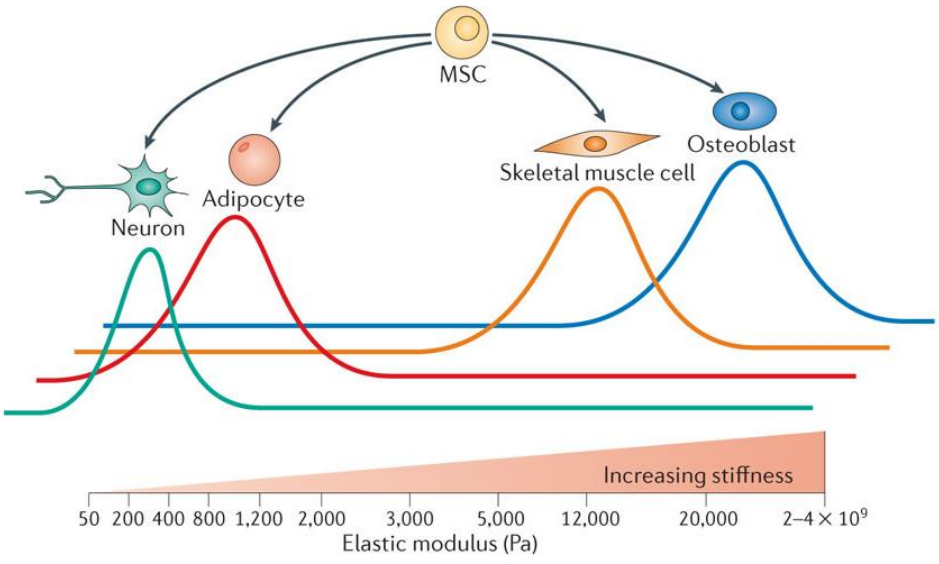


Figure 6: ***Influence of mechanical and physical properties of the ECM on cell behavior****. Cells within tissues experience very different degrees of ECM stiffness, ranging from very soft surroundings (such as those found in the brain and adipose tissue) to very stiff and rigid environments (such as those found within bones or at the bone surface). By recapitulating these different ECM elasticities in vitro, it was found that MSCs differentiate optimally into neurons, adipocytes, skeletal muscle cells or osteoblasts at elasticities that match the physiological ECM stiffness of their corresponding natural niche (shown as colored lines, with peaks indicating maximal differentiation). Pa: Pascal. Adapted from Halder et al. 2012.*

4. Other factors

In addition to the factors described above, there are other – less studied – signals which can influence stem cell fate within the niche.

For example, the topography surrounding stem cells, and in particular in relation to their support cells, has been shown to be a regulator of stem cell proliferation in Drosophila. Briefly, in the germline stem cell niche, the mitotic spindle of dividing cells is oriented, leading to one daughter cell remaining in the niche and self-renewing, while the other exits the niche and differentiates (H. Lin & Spradling, 1995; Yamashita, 2003). Similar observations were made in the control of stem cell self-renewal and differentiation in C. Elegans (Harandi & Ambros, 2015).

In vertebrates, oriented stem cell division has been observed in the skeletal muscle (Kuang, Kuroda, Le Grand, & Rudnicki, 2007), as well as in the epithelium (Lechler & Fuchs, 2005; Seery & Watt, 2000). Similar mechanisms may play a role in other tissues with asymmetric, oriented organizations, like the bone marrow niches, the hair follicle, or intestinal crypts.

Some studies using nanoscale features have revealed how topography can influence MSC differentiation in vitro. Specifically, Dalby et al. were able to induce osteoblastic differentiation in human MSCs using nanoscale disorder and in the absence of osteogenic supplements (García & Reyes, 2005; Polini et al., 2016). Furthermore, in the presence of an osteogenic medium, Engel et al. were able to show a synergistic effect of microstructures on MSC differentiation into mature osteoblasts (Engel et al., 2009). Future studies may reveal new mechanisms at play in these tissues, such as polarization in cell-cell or cell-ECM attachment, or asymmetric growth factor presentation, which regulate these stem cell niches in vivo.

Another example involves the requirement or not of support cells in stem cell proliferation and differentiation. Indeed, while many stem cells require close proximity and interactions with support cells to function or adequately divide and differentiate, there are other cells, such as the intestinal stem cells (ISCs) in Drosophila, which have been shown to

asymmetrically divide in a non-random fashion without the intervention of support cells (Micchelli & Perrimon, 2006; B Ohlstein & Spradling, 2006). During division, the daughter cell in contact with the basement membrane remains an ISC, which the other daughter cell differentiates to become an enteroblast.

Interestingly, this asymmetric division is mediated by the Notch pathway, and while ISCs express Notch ligand Delta, only enteroblasts display an activation of Notch signaling (Benjamin Ohlstein & Spradling, 2007). These observations suggest that ISCs are able to regulate self-renewal through the inhibition of Notch signaling, independently of the intervention of support cells in their vicinity.

II. Stem cell niches in bone

As described above, there are many stem cell niches, and despite similarities in their overall function in terms of stem cell maintenance and differentiation, they remain very different from tissue to tissue. In the context of this thesis, our goal was to investigate differentiation toward an osteoblastic lineage. The main function of differentiated osteoblasts is osteogenesis, the formation of bone extracellular matrix. One of the sites where osteogenesis occurs is in the bone marrow.

1. Composition and characteristic of the bone marrow niche

As depicted in Figure 7, there are two distinct stem cells niches which co-exist and interact within the bone marrow. These niches, namely the hematopoietic and mesenchymal niches, serve very different physiological functions.

The regenerative cells which characterize these two niches are described elsewhere (Reagan & Rosen, 2015), and their differentiation potential is summarized in Figure 8. In the works described below, we were mainly interested in the mesenchymal niche, and specifically the ability of MSCs to differentiate toward an osteogenic lineage.

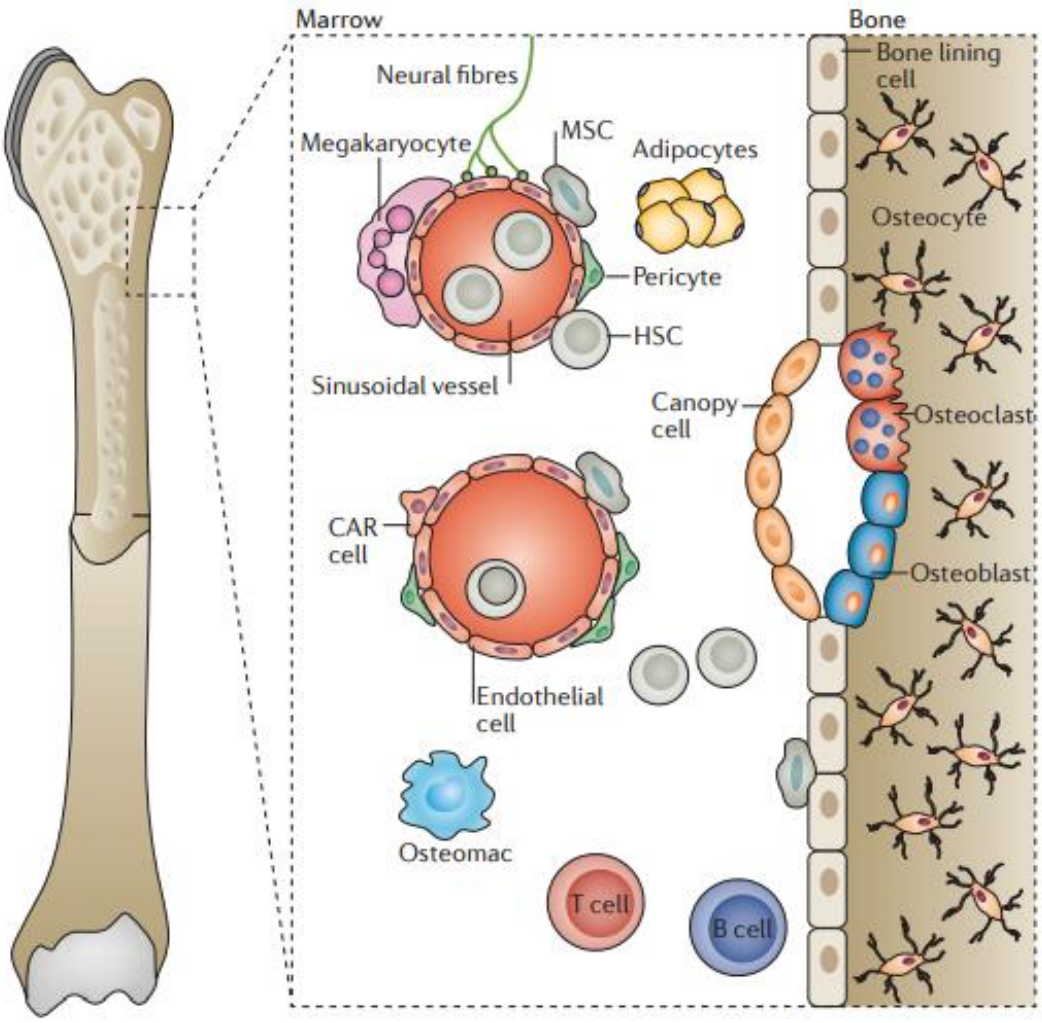


Figure 7: ***The hematopoietic and mesenchymal niches in bone marrow****. The bone marrow niche is composed of multiple cells of mesenchymal and hematopoietic lineages. A hypoxic environment, endosteal bone cells and the proximity of sinusoids and microvessel provide a unique environment for hematopoietic stem cells (HSCs) and mesenchymal stromal cells (MSCs). The union of the skeletal remodeling system and the vascular network within the bone marrow provides a unique niche that regulates whole-body homeostasis. CAR cell, CXCL12-abundant reticular cell. From Reagan and Rosen 2016.*

The hematopoietic niche, which we will therefore be describing only briefly, ensures blood homeostasis through hematopoiesis. Hematopoiesis describes the process by which circulating blood cells are formed from undifferentiated HSCs. As depicted in Figure 8 (Reagan & Rosen, 2015), this process is complex and requires the intervention of many genes, receptors and enzymes which lead HSCs in the bone marrow toward a wide range of fates (Doulatov, Notta, Laurenti, & Dick, 2012). Indeed, the daughter cells of HSCs can be involved in immunity (T, B and NK cells, dendritic cells, monocytes, or granulocytes), oxygen transport (erythrocytes) or blood clotting (megakaryocytes and platelets).

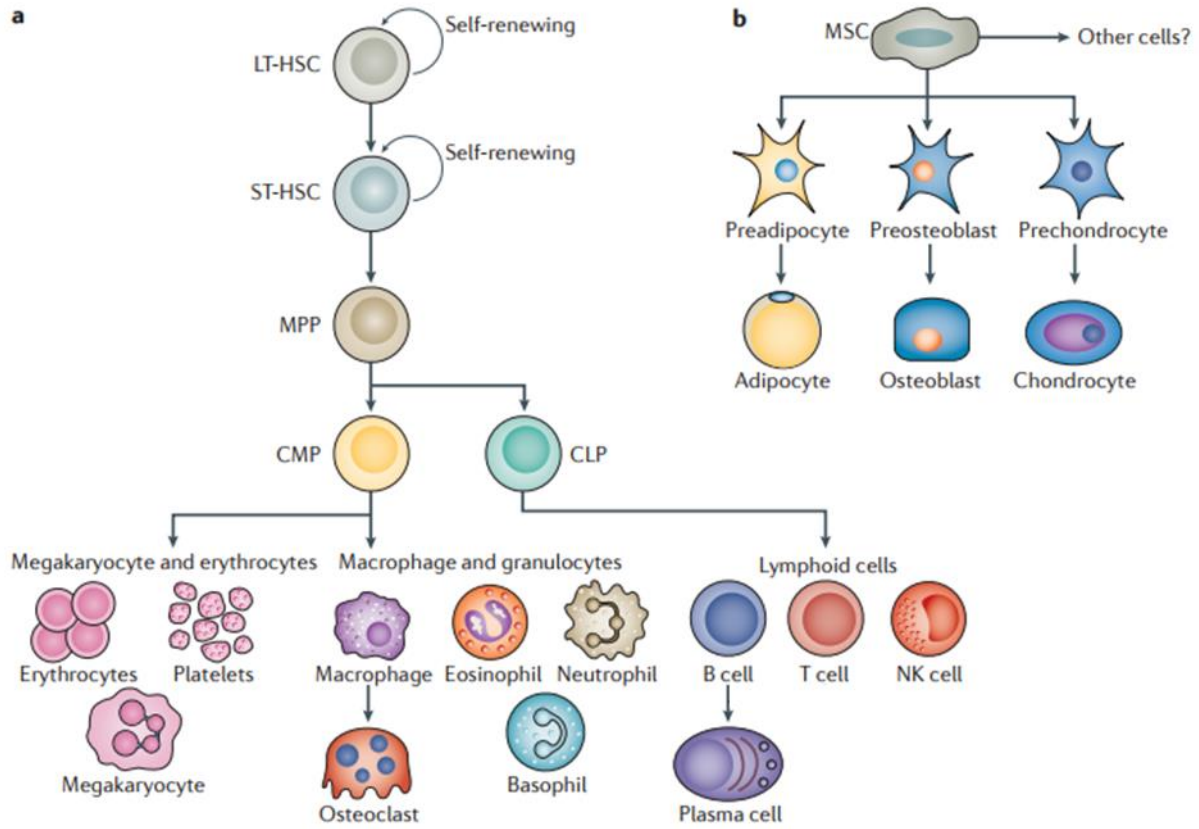


Figure 8: ***The regenerative cells of the bone marrow niche.*** *a. Hematopoietic stem cells (HSCs) give rise to all mature blood cell types. A proportion of these self-renewing cells remain stem cells to maintain a pool of long-term reconstituting HSCs (LT-HSCs) and short-term reconstituting HSCs (ST-HSCs). b. Bone marrow mesenchymal stromal cells (MSCs) are multipotent, self-renewing progenitor cells that can differentiate into other cell types. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MPP, multipotent progenitor; NK cell, natural killer cell. From Reagan and Rosen 2016.*

This ability of a single cell within the HSC niche to transform into such a wide range of daughter cell types is a remarkable reflection of the complexity and precise control of the stem cell niche described above.

While the mesenchymal niche can generate precursor cells for several different differentiated cell lines such as osteocytes, chondrocytes or adipocytes (as depicted in Figure 8), we will mainly focus on its ability to undergo osteoblastic differentiation, and thus generate mature osteoblasts which are responsible for osteogenesis. Osteogenesis is the formation of bone, and occurs not only during bone repair, but also in physiological bone remodeling. This bone remodeling is carried out by temporary structures named basic multicellular units (BMUs), which locally create a specific microenvironment which encourages the coupled intervention of osteoblasts (bone forming cells) and osteoclasts

(bone resorbing cells) (Hauge, Qvesel, Eriksen, Mosekilde, & Melsen, 2001; Raggatt & Partridge, 2010). This microenvironment is shown in Figure 7.

Osteoblasts are obtained through the differentiation of fusiform, fibroblast-like stem cells named mesenchymal stem cells (MSCs), as shown in Figure 8. These pluripotent cells have a known potential for myogenic, adipogenic, chondrogenic and osteogenic differentiation (Bianco, Riminucci, Gronthos, & Robey, 2001; Hentunen, 2008; Rogers, Young, Adkison, Lucas, & Black, 1995). The multipotency of MSCs makes them a useful tool to study the mechanisms and regulators of differentiation, and in cellular therapy (Dominici et al., 2006). Their ability to generate bone tissue has also made them of interest in cell-mediated strategies for bone regeneration (Abdallah & Kassem, 2008).

At first glance, the HSC and osteoblastic stem cell niches seem to serve very different purposes. Nevertheless, their close proximity in the bone marrow has led several teams to investigate possible interactions between these two structures.

A potential role of osteoblasts in hematopoiesis was proposed by Taichman and Emerson in 1998 (Taichman & Emerson, 1998), and confirmed in 2003 by teams led by Li and Scadden. Both these teams showed that osteoblasts act as regulators of the hematopoietic stem cell niche (Calvi et al., 2003; Jiwang Zhang et al., 2003). The effect of the osteoblastic niche on the hematopoietic niche has since been further documented in the literature (Arai et al., 2004; Iwasaki & Suda, 2010; Mansour et al., 2012; Nilsson & Johnston, 2005; Stier et al., 2005). While the HSC niche is rather well defined, both in and of itself, and through its interactions with the osteoblastic niche, the osteoblastic niche remains relatively poorly understood.

2. Osteoblastic differentiation

In vivo, bone is a tissue which is plastic and continuously changing, through physiological maintenance as well as repair. There are three mechanisms that act synergistically to construct and regulate bone tissue: osteogenesis, modeling and remodeling.

These mechanisms are the result of the cooperation of two cell types, namely osteoblasts, which generate bone, and osteoclasts, which resorb it. This process occurs within BMUs (Frost, 1983).

a. Role of osteoblastic differentiation

As described above, osteogenesis is the formation of new bone by osteoblasts. This bone is modeled by osteoclasts to fit within the surrounding physical environment, and then adjusted in response to ever-changing physiological demands (remodeling).

As schematized in Figure 9, once bone remodeling is activated at a specific site, there is the formation of a highly specialized anatomical compartment, namely the bone remodeling compartment (BRC), in which osteoclasts resorb bone, with the concomitant recruitment of osteoblast progenitors, which will differentiate into osteoblasts and synthesize new bone tissue named osteoid (Feng & McDonald, 2011). This osteoid is a pre-calcified bone ECM, which will later undergo mineralization to form mature bone tissue.

The BRC represents a specific microenvironment, composed of pluripotent and differentiated cells, and in which growth factors are embedded in a bone ECM with characteristic mechanical properties. As such, the BRC can be viewed as an osteoblastic niche.

In a broader sense, the biological relevance of this niche and its regulation is highlighted by pathologies resulting from the dysregulation of the osteoblast/osteoclast balance. Indeed, this balance is crucial in the proper maintenance of bone tissue, throughout development and life, and also during bone repair. Excessive bone resorption can lead to pathologies like osteoporosis, whereas excessive bone formation is implicated in diseases like fibrous dysplasia.

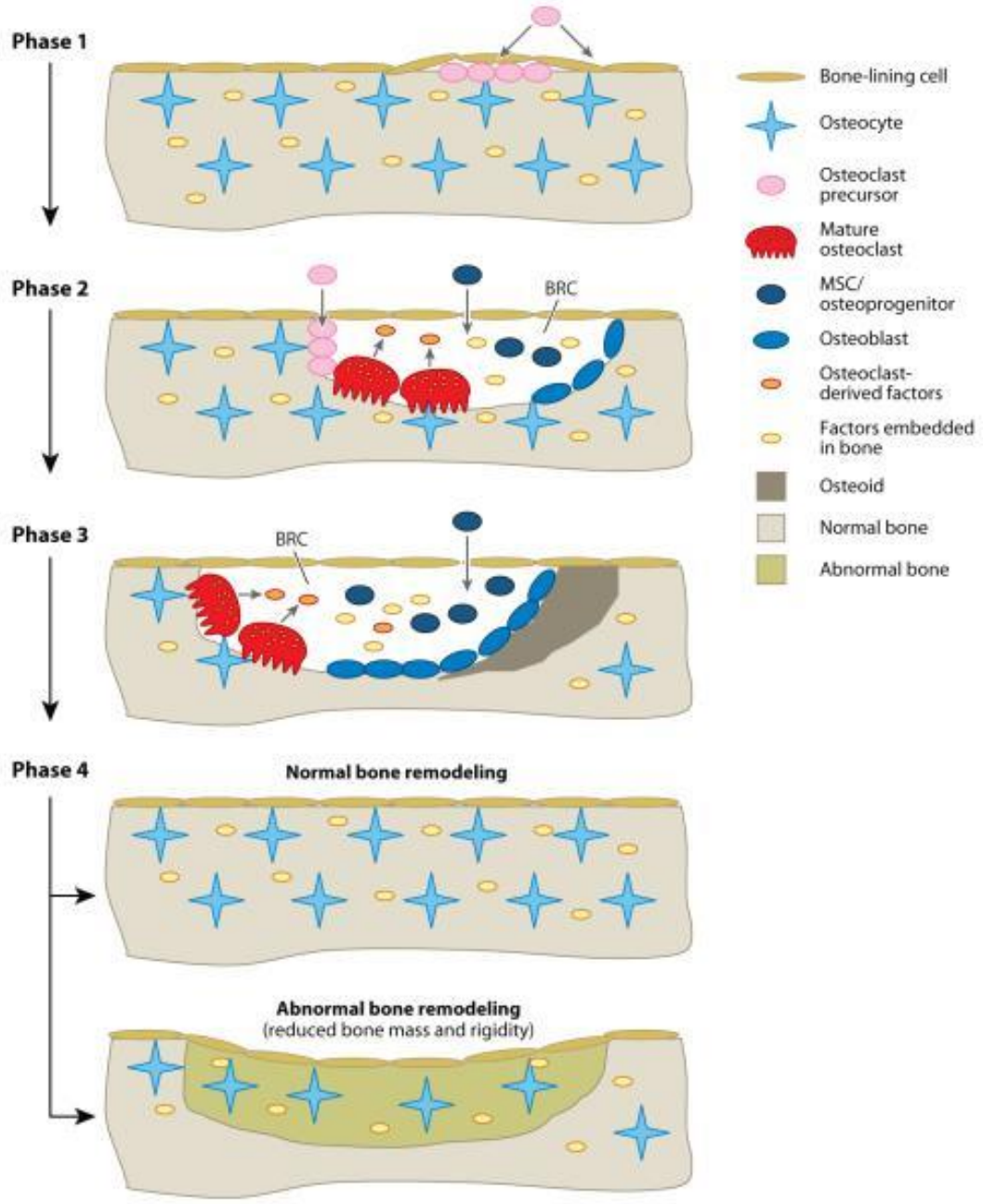


Figure 9: ***Current model for bone remodeling****. The remodeling process consists of four major distinct but overlapping phases: Phase 1: initiation/activation of bone remodeling at a specific site. Phase 2: bone resorption and concurrent recruitment of mesenchymal stem cells (MSCs) and osteoprogenitors. Phase 3: osteoblast differentiation and function (osteoid synthesis). Phase 4: mineralization of osteoid and completion of bone remodeling. In normal bone remodeling, there is no net change in bone mass and strength after each remodeling cycle. However, abnormal bone remodeling in certain pathological conditions, such as osteoporosis, causes reduced bone mass and strength. Abbreviation: BRC: bone-remodeling compartment. From Feng and McDonald, 2013.*

b. Osteoblasts

As the builders of the bone tissue, osteoblasts are a key component of the bone multicellular unit contained in the BRC. Morphologically, these cells are cuboidal, and are localized at the

interface of newly synthesized bone, where they generate an unmineralized matrix which will later mature to become fully formed, mineralized bone tissue.

c. Stages of osteoblastic differentiation

The commitment, and subsequent differentiation of MSCs toward an osteogenic lineage is regulated by many factors, which are described in more detail below.

Master transcriptional regulators have been identified as necessary for lineage commitment (Figure 10), such as MyoD for the myogenesis (Tapscott, 2005), PPARγ2 for adipogenesis (Tontonoz & Spiegelman, 2008), Sox9 for chondrogenesis (De Crombrugghe et al., 2000) and Runx2 for the osteoblast lineage (Komori et al., 1997; Otto et al., 1997).

As is shown in Figure 10, osteoblastic differentiation is a three-stage process which occurs after Runx2 activation:

- In stage 1, the cells keep proliferating, and undergo a Runx-dependent upregulation of fibronectin, collagen, TGFβ receptor 1 and osteopontin.

- The second stage is characterized by the cells exiting the cell cycle and starting to differentiate. During this phase, there is an upregulation of alkaline phosphatase (ALP) and collagen, which results in a maturation of the ECM. At this point, the ECM is called osteoid, and remains immature and unmineralized.

- In stage 3, there is a Runx-dependent upregulation of osteocalcin, osteopontin and collagenase. After collagen, osteocalcin is the second most abundant protein in the bone ECM, and its enrichment in the osteoid promotes the deposition of mineral substance. This mineral substance is composed of calcium phosphate in the form of hydroxyapatite, and provides the bone with its bulk mechanical properties in terms of rigidity and strength (Alford, Kozloff, & Hankenson, 2015; Caetano-Lopes, Canhão, & Fonseca, 2007).

It is during this process that the cell shifts from the fusiform shape of MSCs to the characteristic cuboidal shape of differentiated osteoblasts.

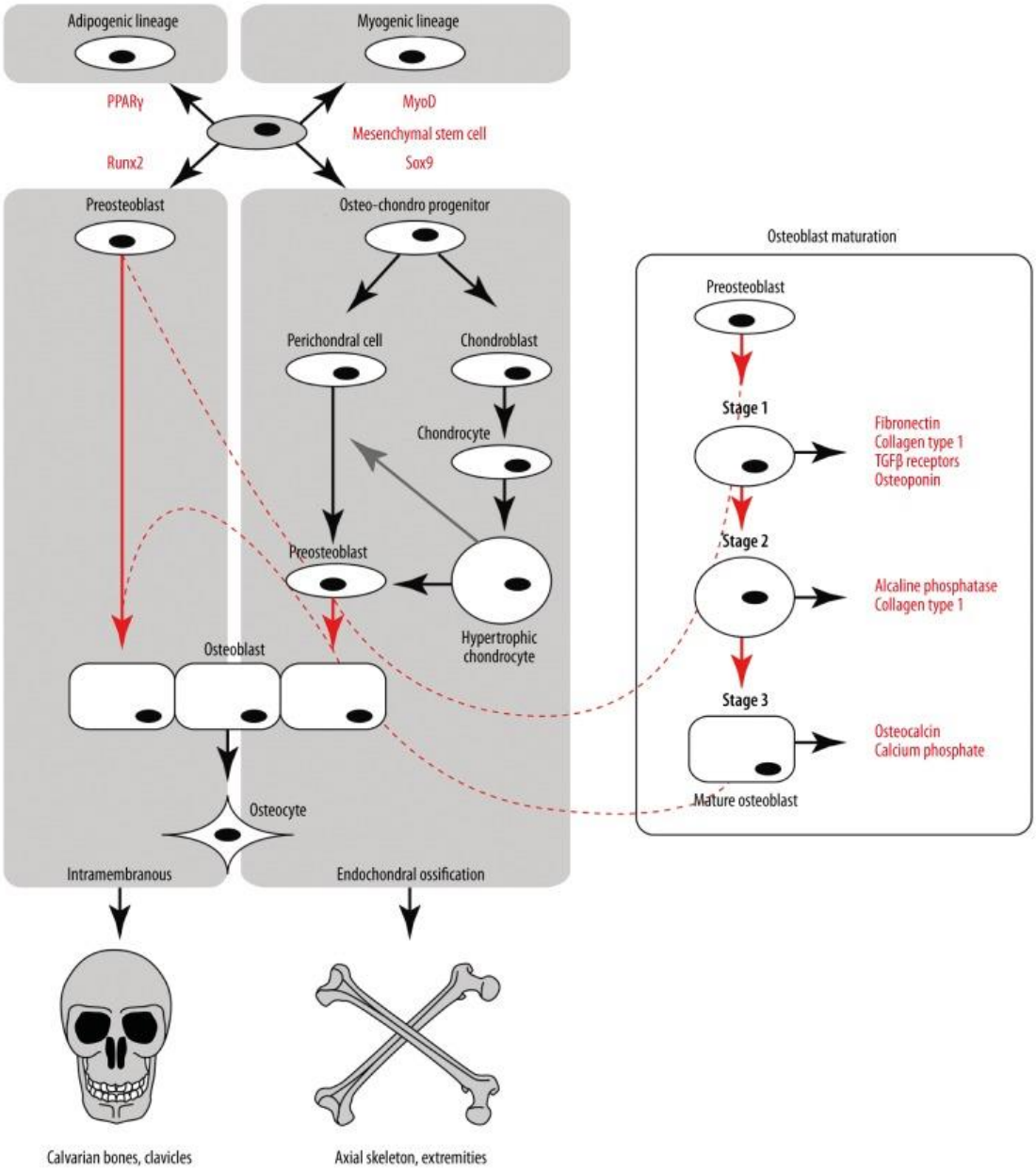


Figure 10: ***A flowchart depicting the biogenesis of osteoblasts****. Mesenchymal stem cells can give rise to 4 lineages (top left) by expressing corresponding transcriptional regulators: PPARg for adipogenic, MyoD for myogenic, Runx2 for osteoblastic, and Sox9 for chondrocytic lineages. In intramembranous ossification (osteogenesis in the scull and clavicles), preosteoblasts stem directly from mesenchymal stem cells, while in endochondral (osteogenesis of the axial skeleton and the limbs) a common osteo-chondro progenitor gives rise to both cell types. Hypertrophic chondrocytes in a paracrine manner (gray arrow) regulate transformation of perichondral cells into preosteoblasts, or might itself transform into one. The process of maturation of preosteoblasts is shown in the enlargement on the right. From Rutkovskiy et al. 2016.*

Although not entirely relevant in the context of this thesis, it should be noted that osteoblasts are not terminally differentiated cells. Indeed, there is a final stage of differentiation, during which two scenarios are possible:

- Osteoblasts that encircle themselves in bone matrix can differentiate into interconnected stellar cells named osteocytes. The function of these cells is not as well characterized as osteoblasts and osteoclasts, but they are thought to play an important role in the regulation of these other cell types thanks to their mechanosensing ability (Lynda F. Bonewald & Johnson, 2008; Lynda Faye Bonewald, 2011; You et al., 2009) and through the secretion of factors like sclerostin (Poole et al., 2005).

- Osteoblasts which remain at the surface of the bone, facing the periosteum, either undergo apoptosis or become bone-lining cells (Hauge et al., 2001), flat surface cells which line quiescent surfaces and close the BRC. Lining cells are believed to have a role in bone resorption, through their production of collagenase, which digests the layer of unmineralized matrix, thus allowing osteoclasts to come in contact with the underlying mineralized matrix (Chambers, Darby, & Fuller, 1985; Chambers & Fuller, 1985).

d. Transdifferentiation

Of note, fully differentiated cells derived from mesenchymal stem cells, such as adipocytes, chondrocytes or myoblasts still present an ability to transdifferentiate into osteoblasts (and vice versa). Transdifferentiation describes the process by which an already committed cell type switches to another cell type, and has been observed and described in many cell-types (Bennett, Joyner, Triffitt, & Owen, 1991; Chelluboina, Dinh, & Veeravalli, 2015; B. Gao, Yang, & Luo, 2014; Katagiri et al., 1994; Pokrywczynska et al., 2015; L. Song & Tuan, 2004).

This ability is of interest for both the understanding of the mechanisms governing stem cell fate determination and the irreversibility or not of lineage commitment, but also from a therapeutic standpoint as a potential resource in cell-mediated regeneration therapies. A

model of some transdifferentiation potentials within MSC-derived cell types is illustrated in Figure 11.

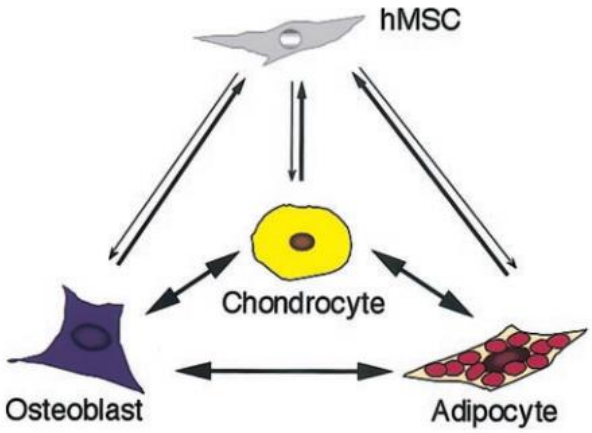


Figure 11: ***A transdifferentiation model of hMSCs****. Osteoblasts, adipocytes, and chondrocytes differentiated from hMSCs were able to transdifferentiate into other mesenchymal cell types. Fully differentiated cells were also capable of dedifferentiation into a primitive stem-like cell type and retention of multiple differentiation potentials. From Song and Tuan, 2004.*

3. The regulation of osteoblastic differentiation

As demonstrated above, osteoblastic differentiation is not a straightforward, linear and clear process. Indeed, while Runx2 is frequently considered as the master regulator of osteoblastogenesis (Gary S Stein et al., 2004), its activity is regulated directly or indirectly by many different factors, and influenced by several signaling pathways. These factors and pathways include Sox9, Msx2, Hedgehog, Osterix, ATF4, NFATc1/Calcineurin, Twist, AP-1, Tcf7/Lef1, Wnt/Notch, Zinc Finger Proteins, and even certain microRNAs. These pathways are complex, tightly regulated, and are known to interact with each other. They are the object of many articles, and their roles in the context of osteoblast differentiation are the object of several reviews (Caetano-Lopes et al., 2007; Jensen, Gopalakrishnan, & Westendorf, 2010; Rutkovskiy, Stensløkken, & Vaage, 2016).

a. Cell-cell interactions and osteoblastic differentiation

In our study, we focused on the mechanisms by which a single, isolated cell was able to undergo osteoblastic differentiation. We used a single cell model to decouple the known

effect of cell-cell interactions on osteoblastic differentiation from other factors influencing stem cell fate. Indeed, intercellular interactions occur within the osteoblastic stem cell niche, and have been shown to regulate the differentiation of MSCs toward an osteogenic lineage, either directly or through synergistic effects with secreted factors. For clear reasons, cell-cell interactions are not directly relevant to the works on single cells described below, so we will be describing only briefly the cell-cell interactions influencing osteoblastic differentiation.

Co-cultures of murine osteoblasts and MSCs in a medium devoid of osteogenic supplements were shown to display an increase in osteogenic gene expression, such as Runx2, type I collagen and osteocalcin in these cells, but only weak osteogenesis (Tsai, Lin, Huang, Lin, & Chang, 2012). While these co-cultured cells also presented increased ALP activity and calcium deposition, all of these markers remained lower than in cells treated with osteogenic supplements.

These results suggest that osteoblasts play a role in the osteoblastic differentiation of MSCs, but that these cell-cell interactions act synergistically with osteoinductive treatment, and are not able to single-handedly induce efficient osteogenesis. Another study using canine MSCs demonstrated a similar effect of osteoblasts in the promotion of osteogenic differentiation (Csaki, Matis, Mobasheri, & Shakibaei, 2009). The mechanisms by which osteoblasts influence MSC behavior were investigated, showing an involvement of the cadherin – β-catenin pathway in the osteoblastic differentiation of MSCs induced by direct cell-cell contact with osteoblasts (Y. Wang et al., 2007).

Osteocytes have also been shown to regulate the osteoblastic differentiation of MSCs. Osteocytes being encased in the bone matrix, they do not influence MSC behavior through direct cell-cell contact, however they do communicate directly with other cells through cytoplasmic processes in bone canaliculi, as shown in Figure 12 (Civitelli, 2008).

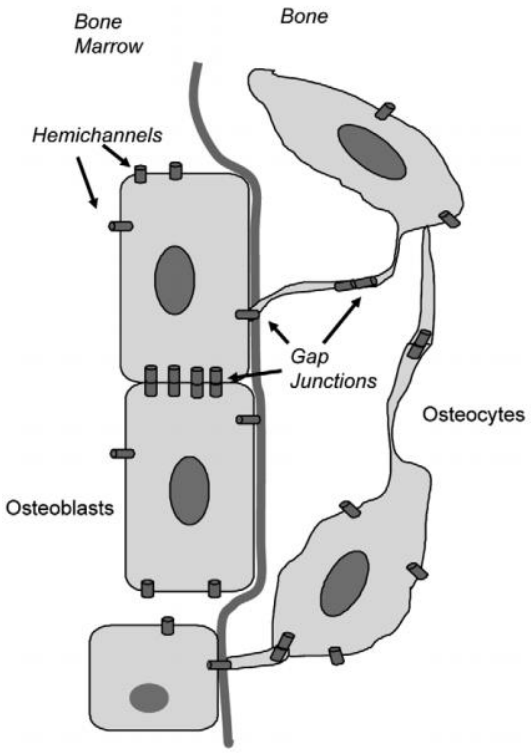


Figure 12: ***Gap junctions and hemichannels in the osteoblast–osteocyte network****. Adapted from Civitelli, 2008.*

These dendritic processes allow osteocytes to communicate with other osteocytes, as well as osteoblasts, through the formation of functional gap junctions (Doty, 1981). This allows osteocytes to regulate osteoblast activity, and thus indirectly influence MSC differentiation. This has been investigated through indirect co-cultures, which demonstrated a synergistic effect of osteocytes and osteoblasts on the osteogenic differentiation of MSCs (Birmingham et al., 2012).

b. Secreted factors

There is a wide range of growth factors and cytokines that have been shown to regulate osteoblastic differentiation. These growth factors include transforming growth factor-β (TGF- β), fibroblast growth factor, platelet-derived growth factor and insulin-like growth factor. Cytokines involved in osteoblastic differentiation include interleukin-1, interleukin-6, and tumor necrosis factor-α. The role of these factors – and others – in osteoblastic differentiation, has been investigated in detail elsewhere (Hughes, Turner, Belibasakis, & Martuscelli, 2006).

As mentioned above, and to facilitate the understanding of the works described below, we will be focusing on the role of the growth factor BMP-2 in osteoblastic differentiation, and in particular its effect on the transcription factor SMAD, and the enzyme ALP, both of which are involved in signaling pathways known to play a role in osteoblastogenesis. BMP-2 is an osteoinductive protein which is part of the bone morphogenetic protein (BMP) family of growth factors.

i. BMP family

BMPs were first reported and described in 1965 and 1971 by Marshall Urist (M. R. Urist & Strates, 2009; Wall & Board, 2014). Currently, 14 BMPs have been identified, 4 of which exhibit high osteogenic activity both in vitro and in vivo (Luu et al., 2007). These BMPs, namely BMP-2, 6, 7 and 9, induce increased ALP activity in vitro, and heterotopic ossification in vivo. Interestingly, BMP-4 was able to induce ALP activity in vitro (H. Cheng et al., 2003), but did not lead to in vivo ossification.

Using recombinant adenoviruses expressing BMPs, Cheng et al. investigated the effect of all 14 members of the BMP family on mesenchymal stem cells, progenitor cells, preosteoblastic and osteoblastic cells. More specifically, they focused on osteogenic activity by measuring the induction of ALP, osteocalcin and matrix mineralization. The data collected led to a better understanding of the role of each protein in osteoblastic differentiation, and to the establishment of a hierarchal model of BMPs, as displayed in Figure 13.

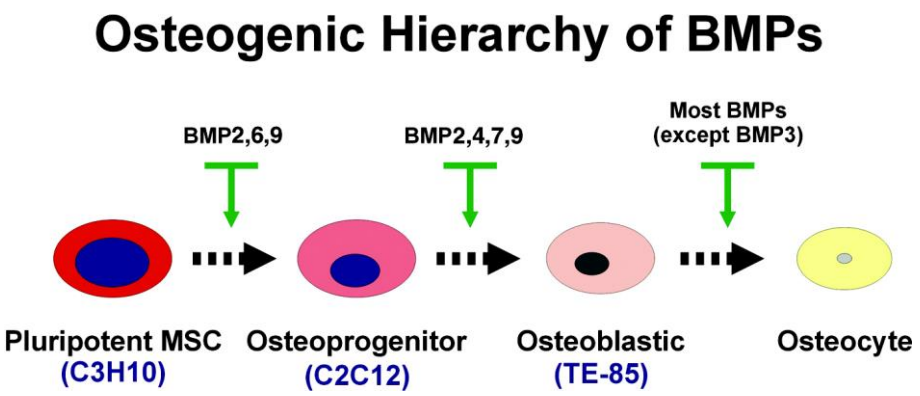


Figure 13: ***Distinct osteogenic activity of human BMPs****. BMP-2, 6, and 9 may be the most potent agents to induce osteoblast lineage-specific differentiation of mesenchymal progenitor cells while most BMPs can effectively promote the terminal differentiation of committed osteoblastic precursors and osteoblasts. From Cheng et al. 2003.*

Of note, the study does not exclude the possibility that combinations of BMPs may also be able to induce osteogenesis in mesenchymal stem cells. It also highlights the key role of BMP-2, the growth factor we used in the works described below, in all stages of osteoblastic differentiation.

From a broader perspective, this physiological relevance of BMPs, and specifically BMP-2, has led to them being widely studied for orthopedic clinical applications to enhance the healing of large bone defects (Conway, Shabtai, Bauernschub, & Specht, 2014; Schmidmaier, Schwabe, Strobel, & Wildemann, 2008), as well as for developing new strategies in bone tissue engineering (Raphael Guillot et al., 2013; Jeon, Song, Kang, Putnam, & Kim, 2007; Shekaran et al., 2014). BMPs are indeed highly potent growth factors (GFs) that play a crucial role in morphogenesis and tissue homeostasis during embryonic development and until adulthood (Hogan, 1996; Kopf, Paarmann, Hiepen, Horbelt, & Knaus, 2014).

With BMP-7, BMP-2 is the only member of the BMP family that is currently used for orthopedic applications. These two BMPs are used in the treatment of spinal fusions, nonunions and oral surgery (Schmidmaier et al., 2008). A comparison of the results in the treatment of long bone nonunion have showed that both BMP-2 and BMP-7 display radiographic healing, and that BMP-2 elicited a higher rate of radiographic healing, achieved radiographic healing more quickly, and finally that these bones were able to bear weight sooner than those treated with BMP-7 (Conway et al., 2014).

ii. BMP-2

This osteogenic activity of BMP-2 in vitro (H. Cheng et al., 2003), as well as its ability to elicit efficient bone formation in vivo (Ogasawara et al., 2004) make it an interesting candidate to study osteoblastic differentiation in vitro.

Structurally speaking, BMP-2 is a homodimer, in which the two monomers are linked by a disulfide bond, as schematized in Figure 14.

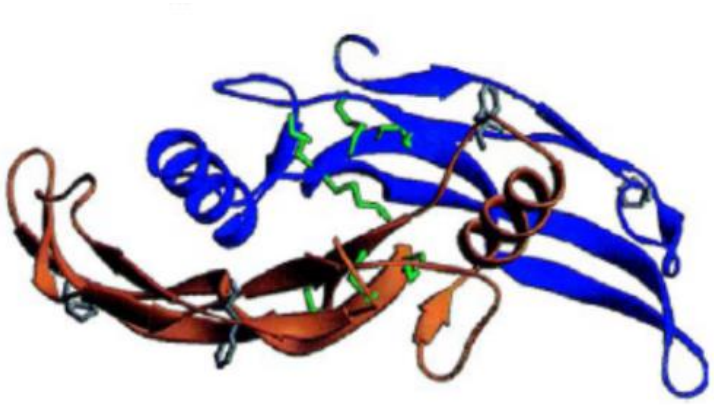


Figure 14: ***3D structure of the native BMP-2 dimer****. α-helices are indicated as spirals, β-strands as arrows, disulfide bridges as green sticks. Adapted from Scheufler et al, 1999.*

Several cell types, including osteoblasts, MSCs and macrophages have been shown to secrete BMP-2, and thus play a role in bone tissue formation (Champagne, Takebe, Offenbacher, & Cooper, 2002; W. Yang et al., 2013). In vitro, BMP-2 has been shown to promote the differentiation of mesenchymal stem cells (MSCs) and osteoblasts toward osteocytes (H. Cheng et al., 2003; Reddi, 1998; Wall & Board, 2014), and induce the transdifferentiation of myoblasts into osteoblasts (Katagiri et al., 1994). In addition, BMP-2 in solution plays a role in early adhesive events, including adhesion and migration through cytoskeletal reorganization (Gamell et al., 2008; a K. Shah et al., 1999).

c. ECM contribution

In additional to cell-cell interactions and growth factors like BMP-2, the composition and characteristics of the ECM play a crucial role in bone formation, and more particularly in osteoblastic differentiation.

Initially, the ECM was thought to serve simply as a structural support for cells, but its role in regulating gene expression, and thus adhesion, proliferation, migration, differentiation or cell death is now better understood (J. C. Adams & Watt, 1993; Allori, Sailon, & Warren, 2008). This is why the study of ECM and its effect on stem cell fate is of great interest in bettering our understanding of the stem cell niche (Votteler et al., 2010).

As mentioned above, the ECM is secreted and remodeled by the cells, and in turn regulates cell behavior through its composition, capacity to store growth factors, and mechanical properties.

i. Composition of bone ECM

The ECM in bone is comprised of inorganic and organic phases, and presents particular properties due to its high mineral content. Indeed, approximately 60% of bone net weight is comprised of inorganic material (Mohamed, 2008). This stiff inorganic phase of bone ECM contains 99% of body calcium and 88% of body phosphate (Gentili & Cancedda, 2009), which is stored in the bone ECM in the form of hydroxyapatite $Ca_{10}(PO4)_6(OH)_2$ crystals.

This inorganic component represents the major part of bone matrix, while the organic component of the ECM represents only 25% of bone net weight. In mature bone tissue, the organic phase consists mainly of collagen – 85-95% of which is type I collagen – and proteoglycans (Gentili & Cancedda, 2009; Mohamed, 2008).

As mentioned above, mineralization of the bone ECM matrix occurs during late osteoblastic differentiation. During the first stages of osteoblastic differentiation, several genes associated with the formation of extracellular matrix are actively expressed by cells, including type I collagen, TGF-β, and fibronectin (G S Stein, Lian, & Owen, 1990). In our experiments, we used fibronectin, a ubiquitous ECM protein associated with cell adhesion, but also osteoblastic differentiation and growth factor retention.

ii. Fibronectin

Fibronectin is a glycoprotein which is widely expressed by many cell types, and mediates a variety of interactions between the cell and the extracellular matrix. As such, it has been shown to be a key player in cell adhesion, migration, growth and differentiation (Pankov & Yamada, 2002; Ruoslahti, 1984).

The role of fibronectin in efficient osteoblastic differentiation and survival, as well as during bone formation, is well-documented in the literature (Bentmann et al., 2009; Globus et al., 1998; Kawelke et al., 2008; A. M. Moursi, Globus, & Damsky, 1997; a M. Moursi et al., 1996). A detailed review of the documented effects of fibronectin on osteoblasts was carried out by Chatakun et al. in 2014, highlighting its effect on adhesion, proliferation, differentiation, or survival, and the mechanisms underlying these phenomena (Chatakun et al., 2014).

Furthermore, fibronectin has been shown to be an organizer of collagen fibrillogenesis, suggesting a potential involvement in ECM maturation in bone (Kadler, Hill, & Canty-Laird, 2008).

iii. GF reservoir

In the osteoblastic niche, as with other stem cell niches, the ECM serves as a reservoir for growth factors (Lind, 1996; M. Urist, DeLange, & Finerman, 1983; Wall & Board, 2014). During the resorption process, these factors are released from the bone matrix, and thus participate in the regulation of osteoblastic differentiation (Feng & McDonald, 2011; Tang et al., 2009).

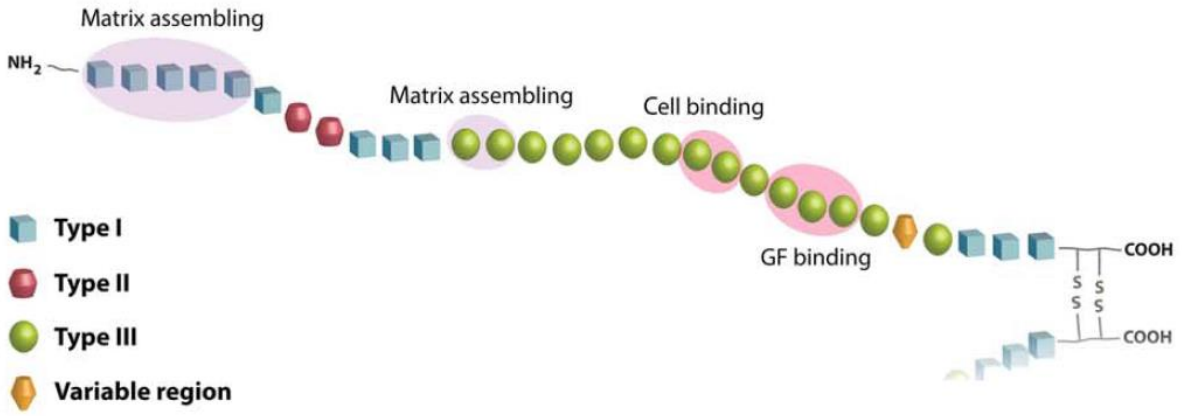


Figure 15: ***Fibronectin-based GF presentation****. FN contains three types of domains that promote integrin binding (III9–10), GF sequestration (III12–14), and FN-FN interactions (I1–5). Adapted from Llopis-Hernandez et al. 2016.*

In the case of fibronectin, specifically, strong interactions between this ECM protein and several growth factors have been identified. The molecular structure of fibronectin, and

specifically its fibrils, has been described in the literature (Früh, Schoen, Ries, & Vogel, 2015). Furthermore, teams led by Hubbell showed highly promiscuous growth-factor domain in the 12^{th}-14^{th} type III repeats of fibronectin, and showed that fibronectin domains can be engineered to promote bone tissue healing (M M Martino et al., 2011; Mikael M Martino et al., 2014). The domains involved in this fibronectin-mediated presentation of growth factors are schematized in Figure 15.

This ability of fibronectin to bind growth factors is relevant in the context of osteoblastic differentiation due to its affinity for BMP-2, as can be seen in Figure 16. Fibronectin can thus present this osteoinductive growth factor in a matrix-bound manner, which is likely to synergistically enhance the intrinsic ability of this ECM protein to induce and promote osteoblastic differentiation.

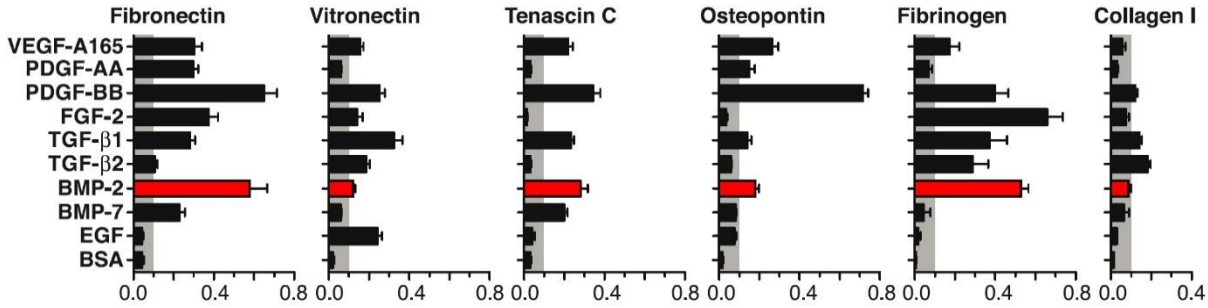


Figure 16: ***GF binding to ECM proteins, measured by enzyme-linked immunosorbent assay.*** *A signal over 0.1 (gray box) was considered to be significant. Bovine serum albumin was used as a control. n ≥ 3 experiments, mean ± SEM. Adapted from Martino et al. 2014.*

iv. Mechanical and geometrical cues

In addition to the factors described above, mechanical cues provided by the ECM have been shown to regulate osteoblastic differentiation (Y.-K. Wang & Chen, 2013). There exists a wide range of mechanisms through which mechanical cues can influence the expression of osteoblast-specific genes (Papachroni, Karatzas, Papavassiliou, Basdra, & Papavassiliou, 2009). The signaling pathways involved in this regulation are summarized in Figure 17.

In the context of this thesis, there are two types of mechanical cues which are relevant to the results observed, namely matrix stiffness and cell spreading.

Matrix elasticity has been shown to influence the fate of stem cells. Indeed, independently of other factors, MSCs grown on matrices of various rigidities displayed differentiation phenotypes in accordance with the tissues these matrices were mimicking (Engler et al., 2006). On soft matrices, MSCs committed to a neurogenic pathway, whereas on stiffer matrices displayed a myogenic phenotype. Finally, on stiff crosslinked-collagen matrices, MSCs favored osteoblastic differentiation.

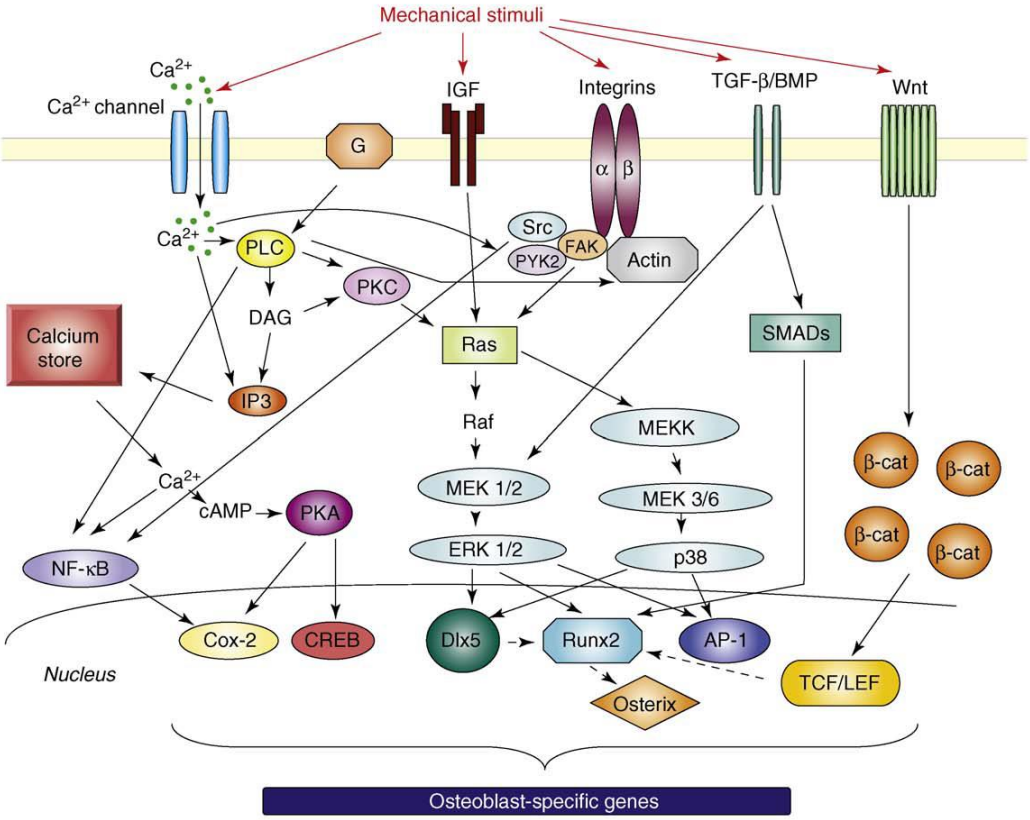


Figure 17: ***Schematic diagram of interactions of different signaling pathways following mechanical stimulation****. Membrane-bound receptors such as Ca2+ channels, integrins, Gproteins, IGF and TGF-b and/or BMP receptors are stimulated by mechanical forces, resulting in induction of several transcription factors that regulate osteoblast differentiation and formation. AP-1 and Runx2 are induced mainly through MAPK and SMADs pathways. Runx2 is also stimulated via the Wnt pathway, involving b-catenin and TCF or LEF factors. PLC–PKA pathway contributes to NF-kB, Cox-2 and CREB induction. Abbreviations: AP-1, activator protein-1; b-cat, b-catenin; DAG, diacylglycerol; FAK, focal adhesion kinase; G, G-protein; IP3, inositol (1,4,5)-trisphosphate; MEKK, MAPK kinase kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PYK2, proline-rich tyrosine kinase 2. From Papachroni et al. 2009.*

Changes in cell spreading – and therefore cytoskeletal tension – have also been reported to play an important role in MSC lineage commitment toward adipogenic or osteogenic lineages (McBeath, Pirone, Nelson, Bhadriraju, & Chen, 2004). Spreading was shown to facilitate osteogenesis, whereas unspreading facilitated adipogenesis. Another study

demonstrated that the inhibition of cell spreading and cytoskeletal tension attenuated BMP-induced osteogenic differentiation in hMSCs (Y.-K. Wang et al., 2012). In both these studies, the RhoA/ROCK pathway was involved, demonstrating the ability of increased cytoskeletal tension, induced by cell spreading, in activating signaling pathways leading to osteoblastic differentiation.

Of note, this regulation is likely mediated by transcription regulators of the Hippo signaling pathway, namely YAP and TAZ. Indeed, these regulators are translocated to the nucleus and activated in highly spread cells, or cells placed on stiff matrices (Halder et al., 2012; Piccolo, Dupont, & Cordenonsi, 2014).

The manners by which cell spreading and ECM stiffness affect YAP/TAZ activity is briefly summarized in Figure 18.

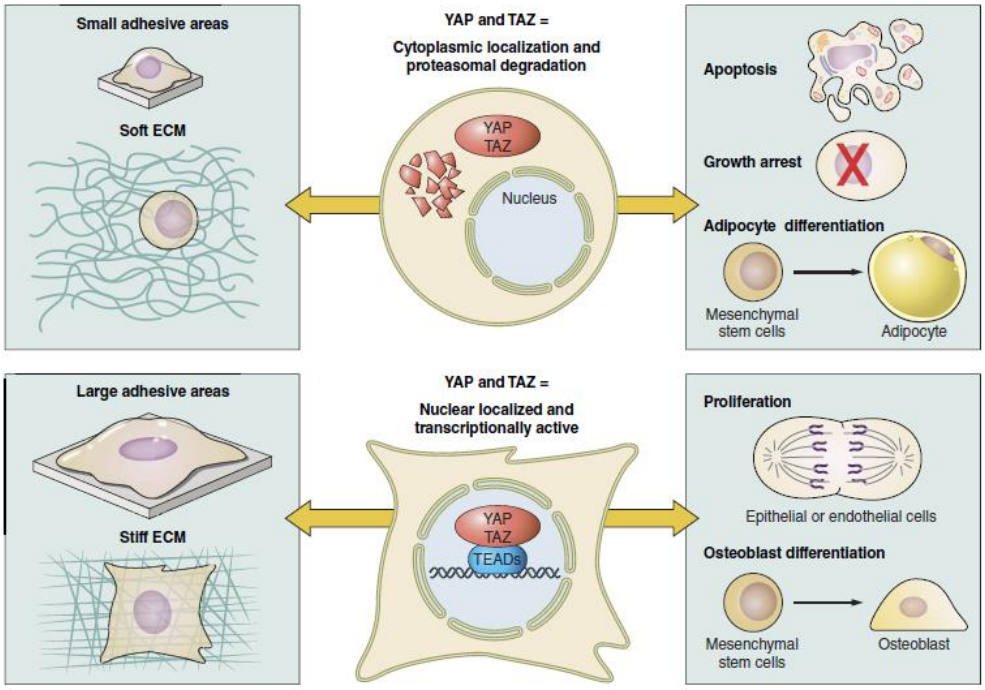


Figure 18: ***YAP/TAZ activity****. Representation of two modalities, cell spreading and ECM rigidity, by which mechanical cues affect YAP/TAZ activity and, in so doing, control multiple cell fate decisions. Adapted from Piccolo et al. 2014.*

Taken together, these results show how mechanical forces can control stem cell fate, and in particular osteoblastic differentiation. The ECM plays a crucial role in this regulation, by

controlling cell spreading and contractility, integrin activation, and substrate stiffness, all of which have been associated to osteoblastic differentiation (Q. Chen et al., 2016). These factors are summarized in Figure 19.

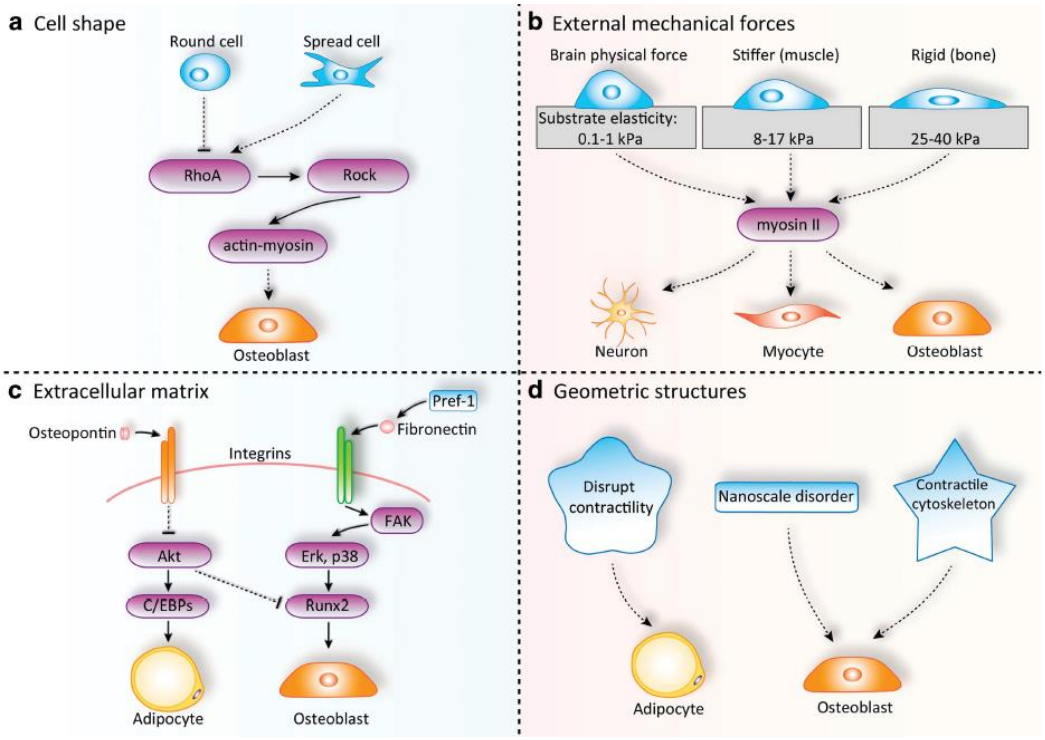


Figure 19: ***Physical factors regulating lineage commitment of MSCs****. MSCs physically interact with various components in the tissue microenvironment in vivo. The physical factors including cell shape, external mechanical forces, extracellular matrix, and geometric structures are involved in stem cell fate decision. By regulating RhoA-ROCK signaling pathway, spread cells tend to differentiate into osteoblasts while round cells tend to become adipocytes (a). Different physical forces can also direct MSCs to differentiate into different lineages via controlling myosin II activity (b). Meanwhile, components of extracellular matrix, such as osteopontin and fibronectin, can regulate the adipo-osteogenic balance of MSCs through binding to integrin receptors (c). In addition, geometric cues such as nanoscale changes can also effectively dictate the differentiation of MSCs (d). From Chen et al. 2016.*

v. Integration of external signals by the cell

1. Cell-cell interactions

Cell-cell interactions have been investigated in the context of osteoblastic differentiation, in particular due to the necessity for cellular condensation during the embryonic formation of bone and cartilage tissues (Hall & Miyake, 1995). In this context, specific transmembrane proteins like N-cadherin and cadherin 11 have been identified as key players in osteoblastic

differentiation, both in vivo (Di Benedetto et al., 2010) and in vitro (Kii, Amizuka, Shimomura, Saga, & Kudo, 2004).

Studies have suggested that while both cadherins play important roles in osteoblastic differentiation, they serve different functions. Indeed, while N-cadherin is present in many mesenchymal cell lines, cadherin 11 appears to be more specifically expressed in preosteoblast and preadipocyte cell lines (Kawaguchi, Kii, Sugiyama, Takeshita, & Kudo, 2001).

This role of cadherin-mediated cell-cell junctions in bone tissue have been described in the literature (S. L. Cheng et al., 1998; Luegmayr, Glantschnig, Varga, & Klaushofer, 2000; Marie, Haÿ, Modrowski, et al., 2014; Marie, Haÿ, & Saidak, 2014). Furthermore, cadherins are connected to the actin cytoskeleton through their binding to α, β and γ catenins (Ratheesh & Yap, 2012; Yonemura, Wada, Watanabe, Nagafuchi, & Shibata, 2010), as shown in Figure 20, and have been shown to play a role in mechanotransduction (DuFort et al., 2011; Orr, Helmke, Blackman, & Schwartz, 2006; N. Wang, Tytell, & Ingber, 2009).

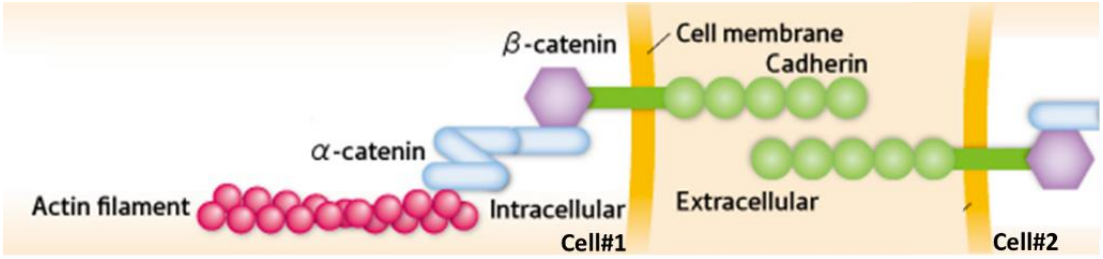


*Figure 20: **The adherens junction**. Cell-cell interactions occur at adherens junctions, through cadherin-mediated interactions. These cadherins are linked to the actin cytoskeleton, and play a role in mechanotransduction. Adapted from Yonemura et al., 2010.*

2. BMP receptors

BMPs are detected by cells through the presence of serine-threonine kinase receptors at the cell membrane. These BMP receptors are divided into two subfamilies, named type-I and type-II, as shown in Table 1 (Nohe, Keating, Knaus, & Petersen, 2004; J. Yang et al., 2014).

Type-I BMP receptors	Type-II BMP receptors
ALK-1 (Acvrl1)	BMPR-II
ALK-2 (ActR-I)	ActR-IIa
ALK-3 (BMPR-Ia)	ActR-IIb
ALK-4 (ActR-Ib)	
ALK-6 (BMPR-Ib)	

*Table 1: **BMP receptors**. ALK: Activin receptor-like kinase; ActR: activin receptor; BMPR: BMP receptor.*

These proteins are composed of an extracellular domain which interacts with receptor-binding motifs on the BMP ligand, a transmembrane domain, and a serine-threonine kinase cytosolic domain (J Massagué, 1998). Both types of receptors are present at the cell surface, and are expressed as homomeric and heteromeric complexes. The type-II BMP receptors are constitutively active, and phosphorylate type-I receptors, thus activating them (Ehata, Yokoyama, Takahashi, & Miyazono, 2013).

For BMP-2, signal induction through BMP receptors has been shown to occur in two distinct manners (Nohe et al., 2002):

- BMP-2 binds to a preformed BMPR-I/BMPR-II homomeric complex, which activates a SMAD-dependent signaling pathways.

- BMP-2 binds to BMPR-I, which in turn recruits BMPR-II, which activates a non-SMAD-dependent pathway resulting in the induction of ALP activity.

Receptor presence and type depends on the cell type, and different BMP ligands bind to specific BMP receptors (Ehata et al., 2013). This is summarized in Figure 21, and ensures the functional specificity of downstream signaling pathways activated by BMP binding. In the context of this thesis, the cell model we used for most experiments was C2C12 myoblasts, for which BMP-2-induced signaling cells has been shown to depend on BMPR-IA and BMPR-II (Heinecke et al., 2009; Mueller & Nickel, 2012; Namiki et al., 1997).

Of note, after ligand binding, BMPR can be internalized through clathrin- and caveolin-dependent pathways, and these different endocytic routes have been shown to influence downstream BMP signaling (Gilde et al., 2016; Hartung et al., 2006). Indeed, we and others have shown that caveolin-mediated internalization is linked to SMAD and ALP signaling, and that clathrin-mediated internalization is only related to ALP signaling (Gilde et al., 2016). We were further able to establish that the amount of internalized BMP-2 depends on the characteristics of the underlying matrix, and in particular to levels of crosslinking. This once again demonstrates the multi-faceted regulation of cell behavior by the ECM, in this case by indirectly affecting the cell's ability to internalize ECM-presented growth factors.

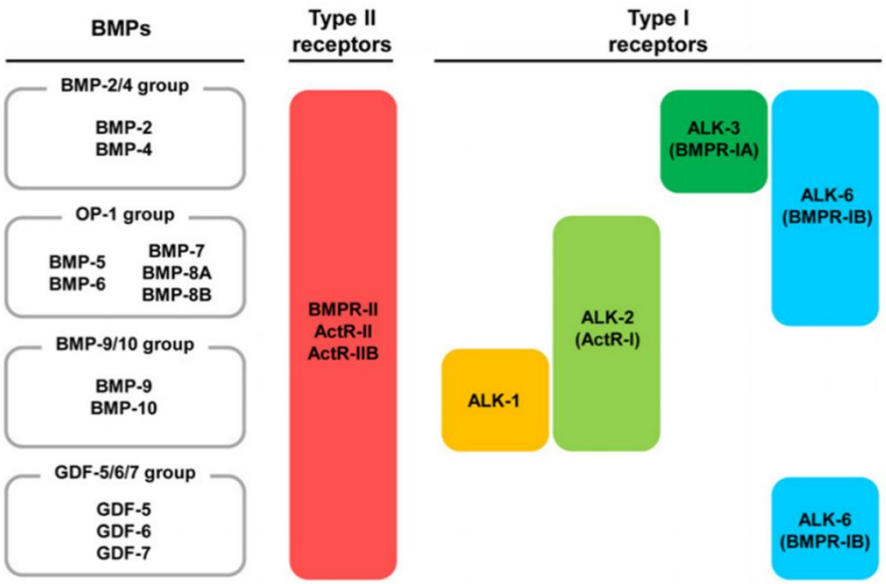


Figure 21: ***Different BMP ligands can bind different type-II and type-I receptors****. BMPs are divided into several subgroups, i.e., BMP-2/4 group, OP-1 group (BMP-5/6/7/8 group), GDF-5/ 6/7 group, and BMP-9/10 group. Adapted from Ehata et al. 2013.*

In addition to their direct effect on BMP signaling, which we will be discussing below, BMPRs may also play an important role in cytoskeletal organization. LIM kinase is a key regulator of actin dynamics, by inactivating the actin depolymerizing factor cofilin. The tail domain of BMPR-II has been shown to interact with LIM kinase, thus suggesting that BMP-2 binding may directly impact the organization of the actin cytoskeleton (Foletta et al., 2003).

3. *BMP-induced signaling pathways*

As mentioned above, the binding of BMP-2 to BMP receptors at the cell surface triggers BMP-induced signaling pathways within the cytoplasm, and this binding is necessary for these growth factors to carry out their biological functions.

a. Non-SMAD-dependent pathway

In the non-SMAD-dependent pathway, BMP-2 binds to the type-I BMP receptor, which in turn recruits the type-II BMP receptor. Type-II BMP receptors activate type-I BMP receptors through phosphorylation, which triggers a mitogen activated protein kinase (MAPK)-dependent signaling pathway.

MAPKs include p38, ERK and PI3 kinase, which are translocated into the nucleus during osteoblastic differentiation, where they regulate the gene expression of osteopontin, ALP or collagen I. BMP-2 has been shown to induce ALP activity in C2C12 cells (Katagiri et al., 1994). Furthermore, this ALP activity, as well as osteocalcin expression, have been associated to the activation of p38 and ERK in these cells, which induces a osteoblastic phenotype after BMP-2 stimulation (Gallea et al., 2001). Similar observations have been made in other cell types, such as adult human MSCs (Jaiswal et al., 2000) and the detailed mechanisms regulating these signaling pathways are well-described in the literature (K.-S. Lee, Hong, & Bae, 2002; Rodríguez-Carballo, Gámez, & Ventura, 2016).

MAPK signaling has been shown to promote MSC differentiation by positively regulating Runx2 and Osterix expression (K.-S. Lee et al., 2002). As shown in Figure 22, TAK1 is activated by BMPR-Ia (Yamaguchi et al., 1995), recruits TAB1, which initiates the MKK-p38 or MKK-ERK1/2 signaling pathways. This promotes the transcriptional activity of Runx2, Dlx5 and Osx, through their phosphorylation by MAPK (M. Wu, Chen, & Li, 2016).

Furthermore, MAPK has also been shown to enhance the canonical SMAD-dependent pathways by phosphorylating Runx2, which promotes the interaction between this master regulator and the SMAD complex (Afzal et al., 2005).

b. SMAD-dependent pathway

In the SMAD-dependent pathway, BMP-2 binds to a preformed BMPR-I/BMPR-2 complex, which results in the phosphorylation of SMAD1/5/8 in the cytoplasm by the type-I BMP receptor. SMAD1/5/8 forms a hetero-trimeric SMAD complex with SMAD4 (Joan Massagué, Seoane, & Wotton, 2005), which decreases the affinity of SMAD1/5/8 for cytoplasmic anchors and increases its affinity for nuclear factors (Shi & Massagué, 2003; L. Xu & Massagué, 2004).

This results in the nuclear translocation and accumulation of the SMAD complex (Hoodless et al., 1996; F. Liu et al., 1996), which crosses the nuclear pore complex through a unique nucleoporin-dependent mechanism (X. Chen & Xu, 2010).

In the nucleus, the SMAD1/5/8- SMAD4 complex recruits co-factors and Runx2 to regulate osteogenic gene expression (Joan Massagué et al., 2005), as schematized in Figure 22.

Of note, it has been shown that this signaling pathway also induces the transcription of Id1, a known inhibitor for myogenesis, suggesting that myogenic inhibition can act synergistically with osteogenic induction to regulate stem cell fate determination (Katagiri et al., 2002).

The two pathways described above are not completely independent nor mutually exclusive, as evidenced by the enhancement of SMAD signaling by MAPK described above. Rather, these pathways cooperate and interact to regulate transcriptional and non-transcriptional aspects of BMP-2-induced signaling (Nohe et al., 2002; Sieber, Kopf, Hiepen, & Knaus, 2009).

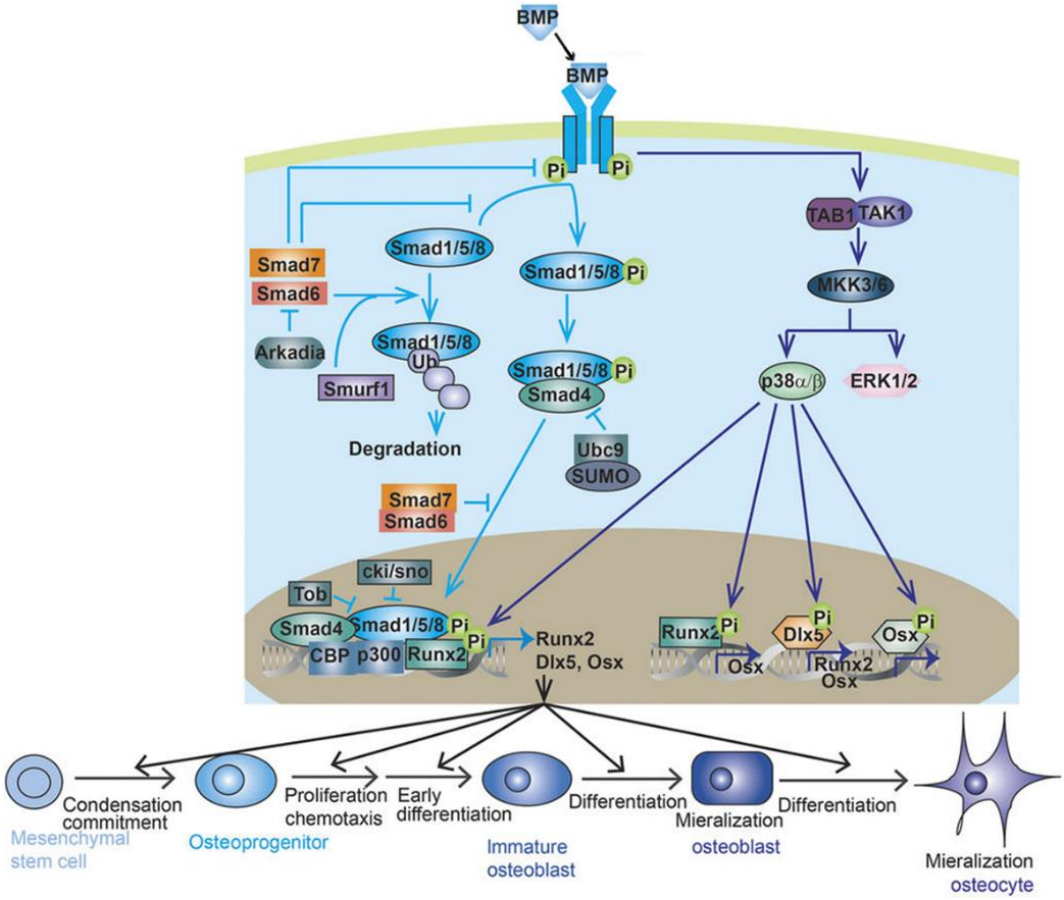


Figure 22: ***BMP signaling in bone****. BMPs bind to homomeric type II receptors, which transphosphorylates homomeric type I receptor to induce SMAD-dependent and non- SMAD-dependent signaling. Adapted from Wu et al. 2016.*

4. Integrins

a. Integrin structure

As mentioned above, cells interact with the ECM through transmembrane receptors at the cell surface named integrins, which are associated to cell adhesion to the extracellular matrix. The combination of the α and β subunits of these transmembrane proteins define the specificity and signaling of an integrin heterodimer. As such, depending on the surrounding ECM, different integrins will be activated, leading to the activation of various downstream signaling cascades.

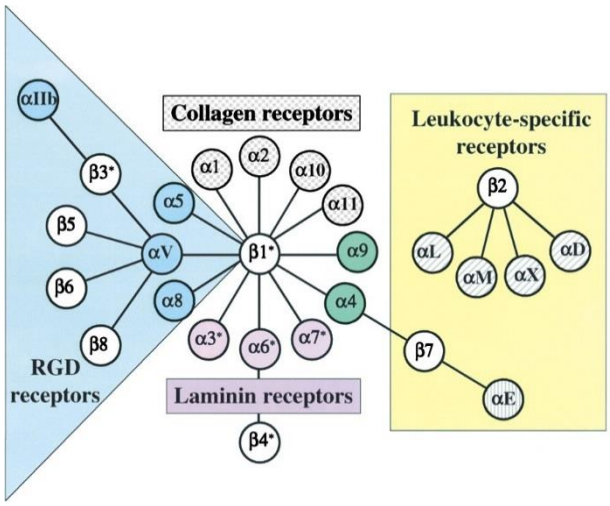


Figure 23: ***The integrin receptor family****. From Hynes, 2002.*

There are several integrin heterodimers that are associated with osteoblastic differentiation (Marie, Haÿ, & Saidak, 2014). The role of these various integrins in osteoblast regulation is summarized in Table 2.

Integrins are comprised of an extracellular, transmembrane and intracellular domain. These domains allow integrin interaction with both the ECM and the cytoplasm, and these interactions are involved in both outside-in and inside-out signaling (Clark & Hynes, 1997; Giancotti & Ruoslahti, 1999; Howe, Aplin, Alahari, & Juliano, 1998; Parsons & Parsons, 1997; Schlaepfer & Hunter, 1998; Schwartz, 1997; Williams, Hughes, O'Toole, & Ginsberg, 1994; Yamada & Geiger, 1997). This bidirectional aspect of integrin signaling is well-documented in the literature (Hynes, 2002; J. Qin, Vinogradova, & Plow, 2004; Schoenwaelder & Burridge, 1999).

Integrin	Ligand	Key cellular pathways	Physiological effect
αvβ1	Osteopontin (RGD)	C/EBPs	Promotes osteoblast differentiation. Inhibits adipocyte differentiation
αvβ3	Fibronectin (RGD)	ILK, ERK1/2	Promotes osteoblast differentiation. Involved in mechanotransduction
α2β1	Collagen 1 (RGD)	ROCK FAK, ERK1/2 Bcl2	Promotes osteoblast differentiation and survival
α4β1	Fibronectin (RGD)	AKT	Promotes osteoblast differentiation and bone formation
α5β1	Fibronectin (RGD)	ERK1/2, BMP-2 IGF1/IGFBP2 PI3K/AKT, Cx43	Promotes osteoblast differentiation and survival, bone formation, and healing. Involved in mechanotransduction

Table 2: ***Summary of key integrin ligands, pathways, and effects in osteoblastic or osteocytic cells****. Adapted from Marie et al. 2014.*

b. Outside-in signaling

Outside-in signaling involves the binding of a ligand to the extracellular domain of an integrin, leading to its activation and the induction of signaling pathways in the cytoplasm. Osteoblastic differentiation and function, for example, have been shown to be regulated in this manner.

Indeed, it has been demonstrated that α5β1 integrin is sensitive to matrix stiffness, and in turn affects osteogenesis through the regulation of cytoskeletal tension (Brunner et al., 2011; Faurobert et al., 2013; T. T. Lee et al., 2015; Marie, Haÿ, & Saidak, 2014). Furthermore, an increase in α5 integrin expression in hMSCs has been shown to induce osteoblastic differentiation (Hamidouche et al., 2009).

αvβ3 integrin, on the other hand, has recently been shown to control SMAD signaling in a stiffness-independent manner in response to matrix-bound BMP-2 (Fourel et al., 2016). This regulation was shown to occur in a Cdc42/LIMK-dependent and RhoA/ROCK-independent manner.

This study suggests a potential crosstalk between integrins and growth factor receptors like BMPR. In the literature, there is strong evidence suggesting other possible interactions between these two cell surface receptors, such as the co-immunoprecipitation of β1 integrins with BMPRs (Lai & Cheng, 2004; North, Pan, McGuire, Brooker, & Kessler, 2015).

Overall, these data suggest that the interaction between integrins and bone ECM plays a crucial role in BMP-2-induced osteoblastic differentiation. These results also suggest that integrins and BMPRs can interact and thus synergistically regulate osteoblastogenesis.

c. Inside-out signaling

Conversely, inside-out signaling has been demonstrated in phenomena like matrix remodeling by the cell. An example of this is FN matrix assembly, as shown in Figure 24 (Singh et al. 2010). Here, the binding of FN to α5β1 integrins leads to their clustering

(outside-in), which ultimately results in ECM remodeling through fibronectin fibrillogenesis (inside-out).

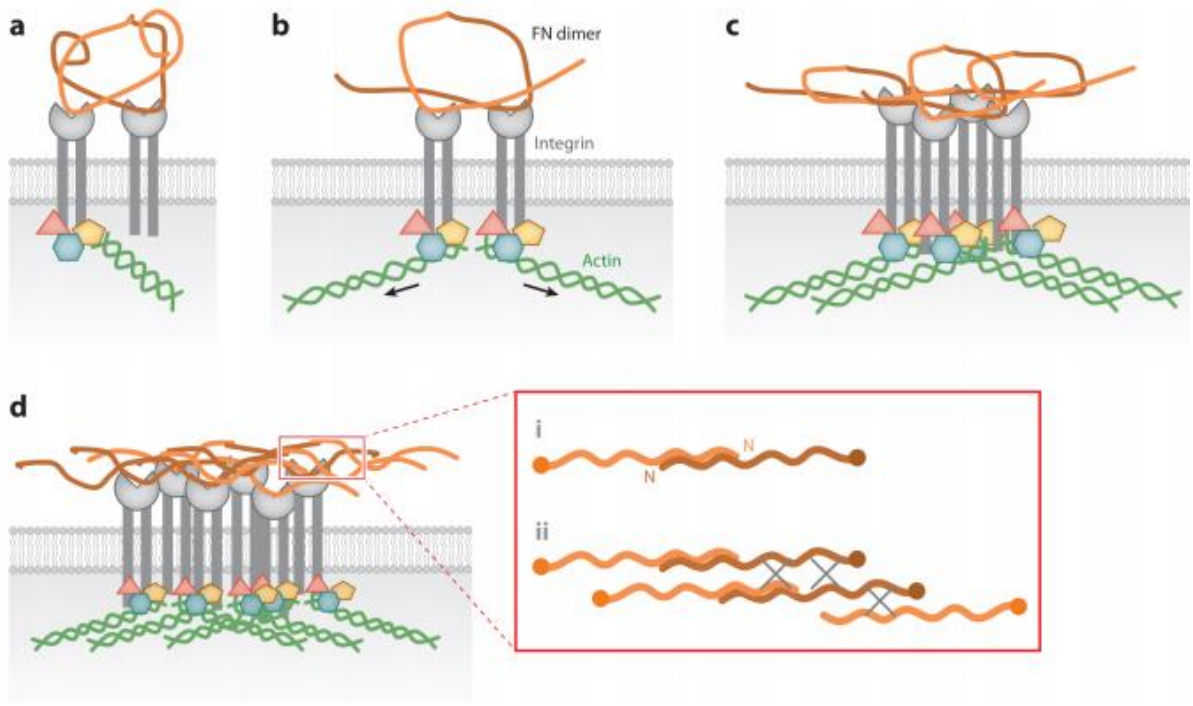


Figure 24: ***Major steps in fibronectin (FN) matrix assembly****. Integrin-induced conversion of compact FN to extended fibrils is shown in four steps. (a) A compact FN dimer binds to integrins (gray). FN subunits of a single dimer are shown in two shades of orange. (b) Intracellular proteins (pink, yellow, blue) are recruited to integrin cytoplasmic domains and connected to the actin cytoskeleton (green). Cytoskeletal connections increase cell contractility (arrows), which induces conformational changes in FN. (c) Integrin clustering and exposed FN binding sites promote FN-FN interactions and further changes in FN conformation. (d) Finally, these events trigger formation of stable insoluble fibrillar matrix. The inset (red box) shows interactions between single subunits of FN dimers. N indicates the N-terminus of an FN subunit. Fibrils form through (i) end-to-end association of FN dimers, mediated by the N-terminal assembly domain, followed by (ii) lateral associations between fibrils that are likely to involve the other FN-binding sites in III1–2, III4–5, and III12–14. Gray X's represent interactions between fibrils. Adapted from Singh et al. 2010.*

5. Vinculin

Integrin activation is known to induce changes in the organization of the actin cytoskeleton, and notably the interaction of F-actin in the cytoplasm with the β subunit of integrin, through the intervention of numerous anchoring proteins (B Geiger, Bershadsky, Pankov, & Yamada, 2001; Kanchanawong et al., 2010; Zaidel-Bar, Itzkovitz, Ma'ayan, Iyengar, & Geiger, 2007). These proteins constitute focal adhesions, dynamic structures linking integrins to the actin cytoskeleton (Benjamin Geiger, Spatz, & Bershadsky, 2009), as shown in Figure 25.

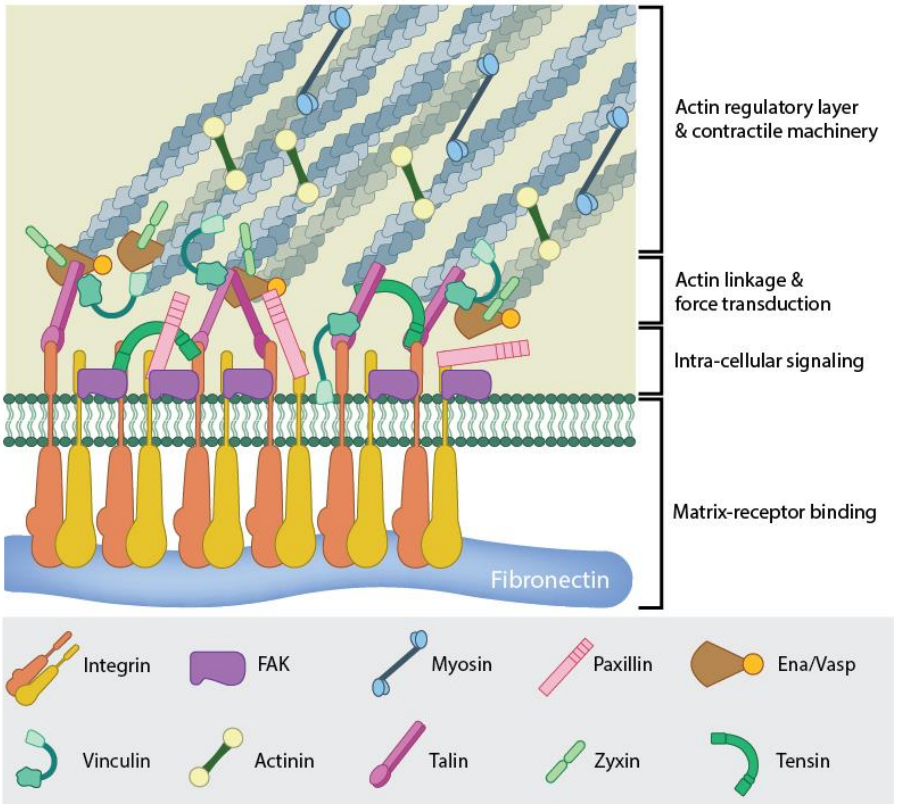


Figure 25: ***Integrin signaling and interactions with the actin cytoskeleton****. From mechanobio.info.*

Vinculin, specifically, is a protein involved in these focal adhesions, and part of the chain linking integrins to the actin cytoskeleton, as shown in Figure 25 (Turner, 2000). Links between the actin cytoskeleton – comprised of F-actin – and certain proteins involved in focal adhesions have previously been described. Indeed, interactions between actin and α-actinin (Blanchard, Ohanian, & Critchley, 1989), talin (Hemmings et al., 1996) or vinculin (Golji & Mofrad, 2013) are already well-documented.

It has also been demonstrated that vinculin interacts with talin (Burridge & Mangeat, 1984; Cohen, Chen, Johnson, Choudhury, & Craig, 2005), and that this interaction is a key player in focal adhesion growth, demonstrated by conformational changes in vinculin after binding of the vinculin head to talin (Fillingham et al., 2005; Humphries et al., 2007) which leads to an increased recruitment of vinculin and talin molecules to the adhesion site. Finally, a transmembrane link between talin and the β1 subunit of integrin has also been established (Horwitz, Duggan, Buck, Beckerle, & Burridge, 1986; Johnson & Craig, 1994).

Taken together, and in the context of osteoblastic differentiation, these data suggest that β1 integrin activation by the ECM may not only enhance BMP-2 signaling, but also induce changes in cytoskeletal organization, which may be linked to the mechanotransduction of external cues from the cell membrane to the nucleus, to regulate gene expression.

6. Transmission to the nucleus

Indeed, forces have been shown to be propagated to the nucleus via integrins and cytoskeletal filaments like actin (Maniotis, Chen, & Ingber, 1997; N. Wang et al., 2009), as shown in Figure 26.

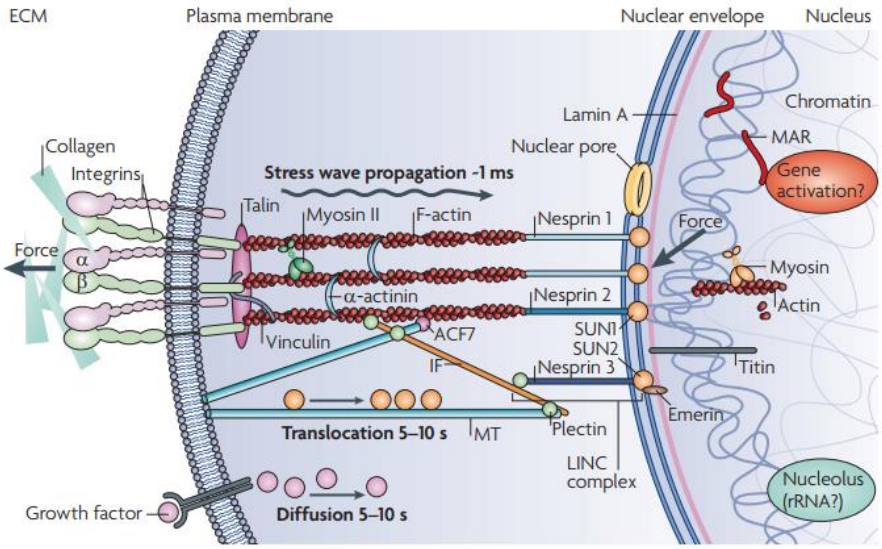


Figure 26: ***Molecular connectivity from the ECM to the nucleus****. A local force applied to integrins through the extracellular matrix (ECM) is concentrated at focal adhesions and channelled to filamentous (F)-actin, which is bundled by α-actinin and made tense by myosin II, which generates prestress. F-actins are connected to microtubules (Mts) through actin-crosslinking factor 7 (AcF7), and to intermediate filaments (iFs) through plectin 1. Plectin 1 also connects iFs with Mts and iFs with nesprin 3 on the outer nuclear membrane. Nesprin 1 and nesprin 2 connect F-actin to the inner nuclear membrane protein SUN1; nesprin 3 connects plectin 1 to SUN1 and SUN2. Owing to cytoplasmic viscoelasticity, force propagation from the ECM to the nucleus might take up to ~1 ms. The SUN proteins connect to the lamins that form the lamina and nuclear scaffold, which attaches to chromatin and DNA (for example, through matrix attachment regions (MARs)). Nuclear actin and myosin (and nuclear titin) might help to form the nuclear scaffold, control gene positioning and regulate nuclear prestress. The force channeled into the nuclear scaffold might directly affect gene activation within milliseconds of surface deformation. By contrast, it takes seconds for growth factors to alter nuclear functions by eliciting chemical cascades of signaling, which are mediated by motor-based translocation or chemical diffusion. LiNC, linker of nucleoskeleton and cytoskeleton; rRNA, ribosomal RNA. Adapted from N. Wang, Tytell, and Ingber 2009.*

At the nuclear envelope, the LINC (LInker of Nucleus and Cytoplasm) complex connects the nucleus to the cytoplasm. The LINC is comprised of nesprins, SUN and lamin proteins (Crisp et al., 2006; Haque et al., 2006; Padmakumar et al., 2005). Emerin is a protein which is present at the inner nuclear membrane, and which interacts with the LINC complex through nesprins and lamins (Mislow et al., 2002; Wheeler et al., 2007). It is part of the LEM (LAP2-emerin-MAN1) domain protein family, which is characterized by its ability to bind lamin. The LINC complex is involved in mechanotransduction to the nucleus, as evidenced by the fact that a deficiency in its proteins has been shown to affect mechanical coupling (Lammerding et al., 2004; Rowat, Lammerding, & Ipsen, 2006).

Of note, the presence of lamin A and LEM domain proteins has been shown to modulate signals from the BMP signaling pathways. In preosteoblastic cell lines, for example, BMP-2-induced osteoblastogenesis led to an upregulation of lamin A/C expression (Tsukune et al., 2017). Another study identified SMAD1 Antagonist Effector (SANE), a LEM domain protein which has an effect on BMP signaling (Raju, Dimova, Klein, & Huang, 2003). In C2C12 myoblasts, SANE inhibited BMP signaling, as demonstrated by the inhibition of alkaline phosphatase induction. SANE was shown to bind to SMAD1/5 and BMP type I receptors, thus regulating BMP signaling.

Taken together, all the elements described above show the complexity of stem cell niches in general, and the osteoblastic niche in particular, in regulating stem cell fate. This complex machinery involves the integration of a myriad of external cues provided by the local environment of the cell, which regulate intrinsic signaling within the cell.

While some of these cues originate from cell-cell interactions in the osteoblastic niche, many are provided by the ECM. Studying specific ECM-induced effects on osteoblastic differentiation without interference from cell-cell interactions would be rather difficult in vivo. This is particularly true considering the wide range of growth factors, receptors and signaling pathways which are involved in this niche. These elements show that there is an undeniable need to develop and use engineered extracellular matrices in order to simplify,

or at the very least control, the external factors being presented to the cell, and how each one of these factors impacts cell behavior.

III. Engineering the stem cell niche

The elements described above highlight the importance of the ECM in stem cell fate determination, and in particular in osteoblastic differentiation. The ECM participates in the regulation of the stem cell niche, by modulating the self-renewal, maintenance, proliferation and differentiation of stem cells. This control is exerted both directly and indirectly, through the ECM's intrinsic composition and mechanical properties, as well as its function as a reservoir for growth factors.

This multi-faceted regulation of stem cell behavior by the ECM reveals the necessity of engineering biomaterials to mimic in vivo conditions, and to decouple the effect of each component on cell behavior in order to further our understanding of how this regulation takes place.

1. Approaches to studying the cell microenvironment

The ideal way to investigate stem cell response to its microenvironment would be to directly study the stem cell niche in vivo. For example, regarding the ECM, there are teams which have demonstrated the role of collagen and fibronectin in muscle regeneration in mice (Bentzinger et al., 2013; Urciuolo et al., 2013). However, the complexity of the ECM and its diverse roles in cellular processes make in vivo analysis very difficult, and render signal decoupling almost impossible.

Indeed, there are two strong challenges with the in vivo study of the ECM.

Firstly, the depletion or overexpression of an ECM component may result in changes in signaling pathways that are indirectly induced by that component. For example, depleting fibronectin expression in a tissue will impact integrin-mediated signaling, but it will likely also affect the mechanical properties of the tissue, as well as the ECM's ability to retain

certain growth factors and present them to the cells. In these conditions, correlating changes in cell behavior with one of these aspects of fibronectin-induced signaling is very challenging.

The second issue with in vivo studies is potential compensatory and redundant mechanisms existing in these complex systems. An example of this type of compensation was demonstrated in mice, in which the loss of the sarcoglycan complex, a protein complex linking the cytoskeleton to the ECM in muscle, was shown to be compensated by the upregulation of integrin α7β1 (Allikian, Hack, Mewborn, Mayer, & McNally, 2004). The true role of ECM components on cell behaviors like adhesion or differentiation can thus be masked by these mechanisms.

On the other end of the spectrum, there are many studies investigating factors influencing cell behavior using off-target substrate rigidities or only soluble factors, dissociated from the ECM. The advantage of placing growth factors like BMP-2 in solution is their ease of use. They also allow the precise characterization of cell responses to these factors (Aoyama et al., 2011; Gorskaya et al., 2013; Katagiri et al., 1994), dose-responsiveness (Fiedler, Röderer, Günther, & Brenner, 2002), or even synergistic effects of combinations of growth factors (Kémoun et al., 2007; Knippenberg, Helder, Zandieh Doulabi, Wuisman, & Klein-Nulend, 2006; X. Lin, Zamora, Albright, Glass, & Peña, 2004; W. Zhu et al., 2006). Despite its convenience for rapid cell culture assessment in culture plates, studying BMP-2 in this fashion presents several limitations. First of all, the lifetime of BMP-2 in solution is less than 24 hours, but is significantly improved when the presentation mode is changed (Takada et al., 2003). This can be a problem for long term studies, requiring the constant renewal of BMP-2 in the cell culture medium. Secondly, plating cells on culture plates inevitably implies that they are exposed to limited signaling from the ECM, obtained from their own deposition of ECM proteins or the presence of adsorbed serum components. This does not allow us to determine the contribution of ECM-mediated cues on cell behavior. This is notably true for matrix stiffness, considering the supraphysiological rigidity of the plastics or glass used for cell culture.

To resolve this issue, there exists a wide range of intermediate approaches to study the cell's environment, useful tools which can be tailored to best respond to the requirements of a specific study. Depending on the biological question, the approaches to engineering a tissue can be based on reductionalist or organotypic models, as well as models that attempt to bridge the gap between the two, as shown in Figure 27.

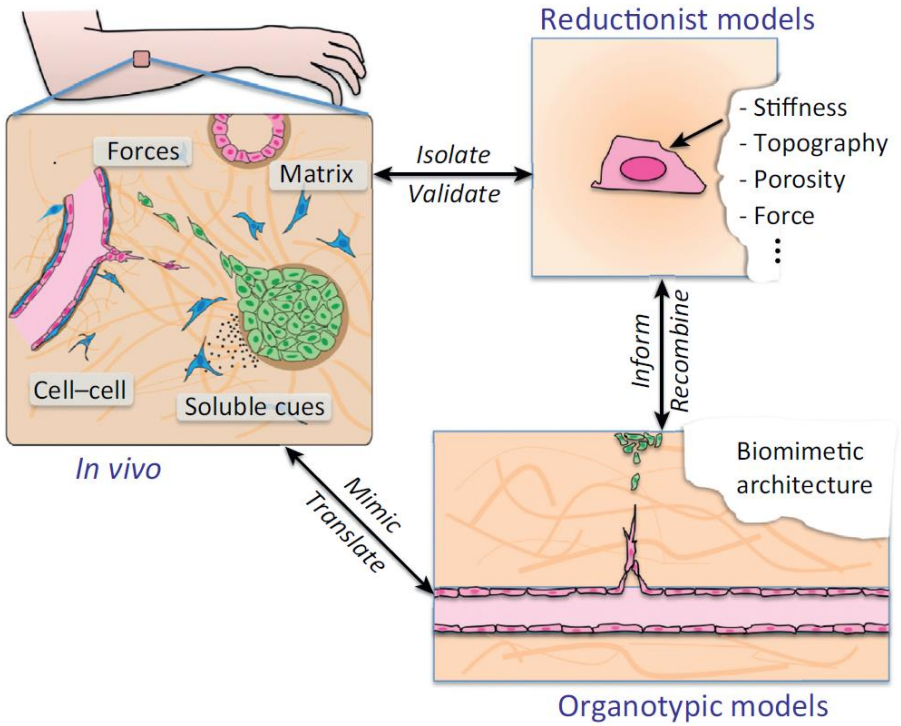


Figure 27: ***In Vivo and In Vitro Models Have Coevolved Synergistically to Provide Distinct Approaches to Understanding Living Systems****. New biomimetic models offer the potential to provide a third approach to the ecosystem, reconstituting more complex behaviors in culture. From Chen, 2016.*

Organotypic approaches attempt to mimic the in vivo environment as closely as possible.

Examples of this include the use of organ slices, which are extracted from live tissue then cultured in vitro. These are used for physiological, toxicological, or pharmacological studies (Morin et al., 2013; Parrish, Gandolfi, & Brendel, 1995). These types of cultures have been carried out in tissues from many different organs, including the lung (Morin et al., 2013), liver (Lerche-Langrand & Toutain, 2000), kidney (Catania et al., 2003) or brain (Merz et al., 2013).

When studying the specific role of the ECM, this approach can involve decellularizing a tissue or organ, thus preserving only the native ECM. This ECM is still able to guide stem cell fate, as evidenced in the rhesus monkey kidney (Nakayama et al., 2010), and can be used for tissue and organ engineering (J. J. Song & Ott, 2011; Soto-Gutierrez et al., 2010).

Reductionist approaches are the most frequently described in the literature, through the 2D culture of cells on glass or plastic plates, in the presence or not of soluble factors influencing their behavior.

Organotypic approaches are highly complex, due to the presence of many signals influencing cell behavior. They are also sometimes difficult to image, due to technical limitations relative to their thickness. Furthermore, they require the excision of entire organs and tissues, which implies cost and ethical considerations limiting their use. Reductionist approaches, due to their simplicity and ease of use, allow the precise characterization of certain factors, but inevitably ignore many key functions and signals which are present in vivo. As such, they allow precise characterization of factors, but not the comprehensive understanding of mechanisms occurring within a tissue.

These reasons have led to the development and engineering of models bridging the gap between organ and single cell. These different categories of cell culture have been reviewed by Shamir and Eswald in 2014, and are summarized in Figure 28 (Shamir & Ewald, 2014).

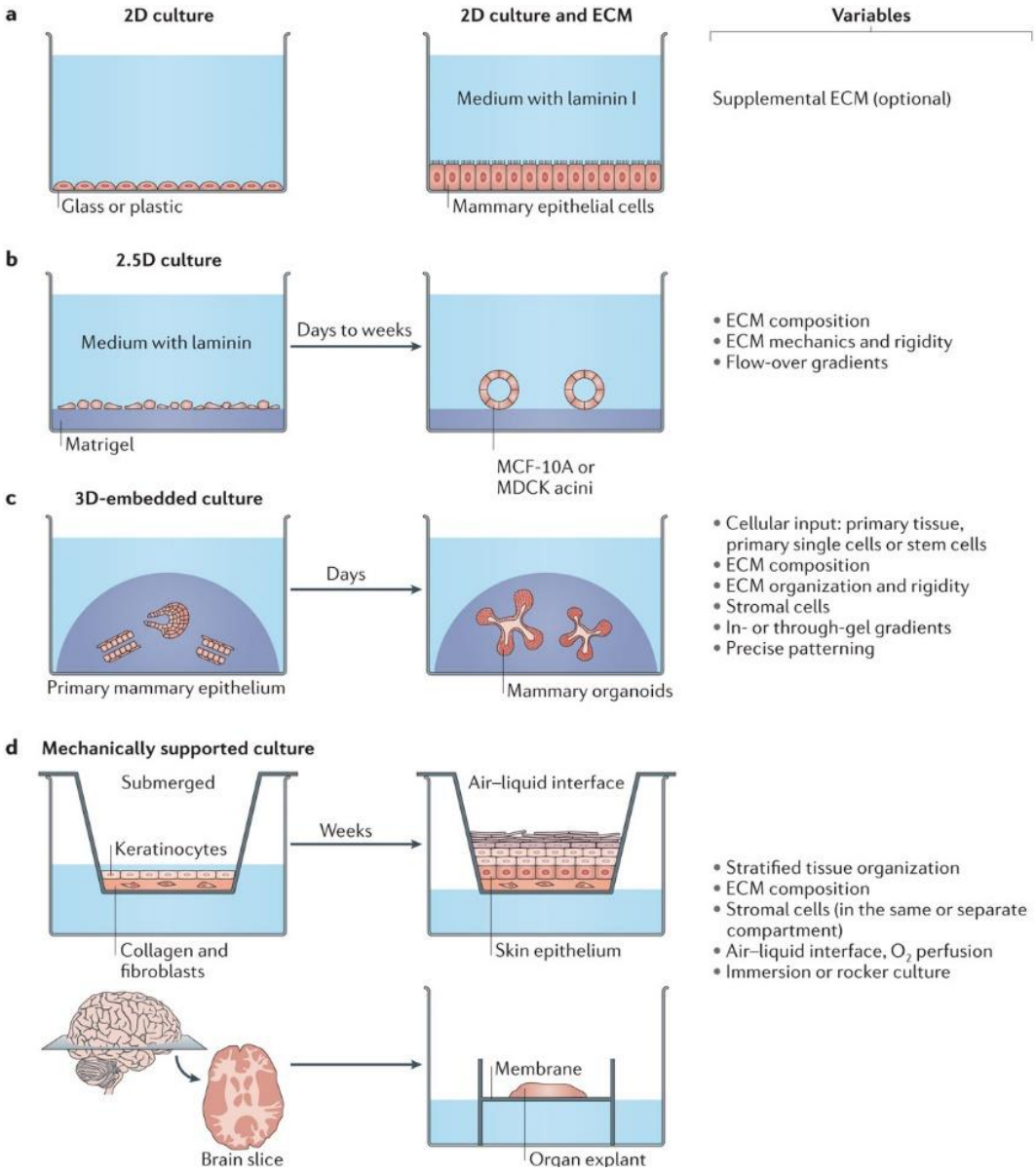


Figure 28: ***The major categories of cell culture****. From Shamir & Ewald 2014.*

From an engineering standpoint, there are several tools that can be used to investigate the effect of soluble ligands, cell-cell interactions, cell-ECM interactions and other parameters like pH, mechanical or electrical stimuli (Gattazzo, Urciuolo, & Bonaldo, 2014). Some of these tools are shown in Figure 29.

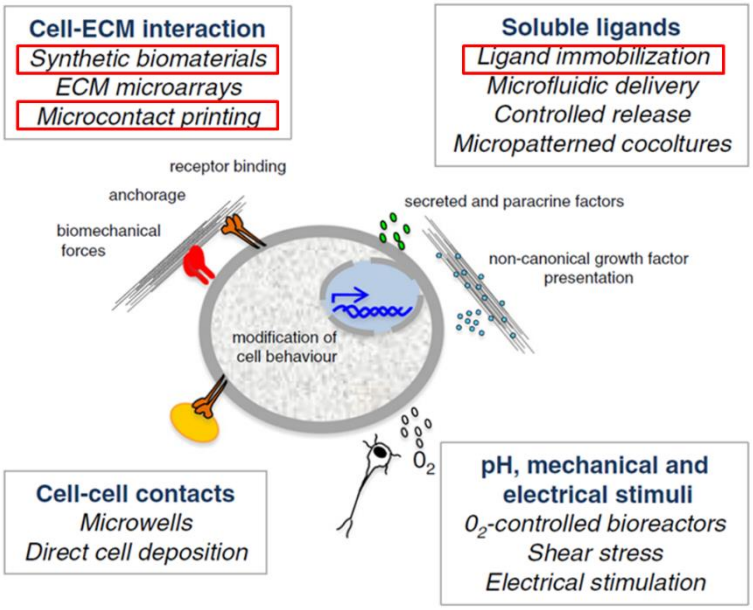


Figure 29: ***Strategies for engineering stem cell niches****. (A) Schematic representation of the engineering techniques used to reproduce the chemical, physical and mechanical microenvironment of the stem cell niche. The strategies used in the works described below are framed in red. From Gattazzo et al. 2013.*

In the context of this thesis, we used a type of 2.5D culture as a tool to recreate in vitro some aspects of MSC behavior, and in particular the in vivo induction of osteoblastic differentiation. We investigated the effect of the ECM using engineered biomaterials, the effect of cell shape using microcontact printing, and the effect of growth factor presentation using ligand immobilization (Figure 29). Depending on the experiments, and in particular the need for force measurement using traction force microscopy (TFM), we used either polyacrylamide gels or polyelectrolyte multilayer films as soft biomimetic substrates.

2. Polyacrylamide gels

Hydrogels of polyacrylamide (PAA) are frequently used as engineered ECMs due to their low cost and useful properties. Indeed, PAA gels are optically transparent and composed of chemically simple compounds. Their mechanical properties are dictated by the monomer/crosslinker (acrylamide/bisacrylamide) ratio, and their stiffness can thus easily be adjusted through simple changes in solution preparation (Amini, Yan, & Sun, 2011; Denisin & Pruitt, 2016; Engler, Richert, Wong, Picart, & Discher, 2004; Pelham, 1998).

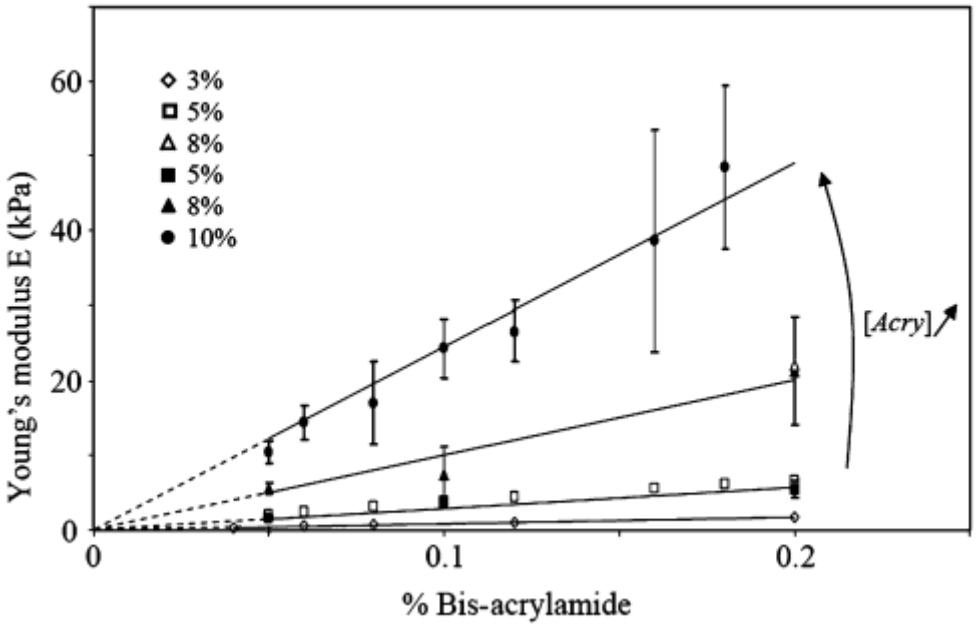


Figure 30: ***Comparison of measured and reported elastic properties of polyacrylamide gels as a function of acrylamide and bis-acrylamide concentrations and with different techniques****. Filled symbols correspond to the measurements of the study, while open symbols indicate data collected from the literature. Error bars represent mean ± SD. From Boudou et al. 2006.*

This characteristic of PAA gels has led to them being used to study the effect of matrix rigidity on osteoblastic differentiation in several cell types, including MC3T3 preosteoblasts (Mullen, Vaughan, Billiar, & McNamara, 2015), human MSCs (J. S. Park et al., 2012) or human bone-derived cells (Witkowska-Zimny et al., 2013).

These properties, combined with the fact that PAA can easily be used to generate micropatterns using photolithography, as described below, make it a promising candidate to study the effect of the ECM on stem cell behavior (Vignaud, Ennomani, & Théry, 2014). Furthermore, fluorescent beads can be incorporated into the PAA gels prior to polymerization, thus making it possible to track by TFM the mechanical forces exerted by the cell on its underlying substrate (Dembo & Wang, 1999; Gupta, Doss, Lim, Voituriez, & Ladoux, 2016; Legant et al., 2013).

3. Polyelectrolyte multilayer films

Polyelectrolyte multilayer films are obtained by the layer-by-layer deposition of polyanions and polycations on a substrate. This layer-by-layer assembly process was first described by

Decher et al. (G. Decher, Hong, & Schmitt, 1992; Gero Decher, 1997; Lvov, Decher, Haas, Möhwald, & Kalachev, 1994), and has since been developed and used by many other teams, due to the versatility and ease of use of the technique, in particular for biomedical applications. This use for biomedical applications is allowed by the fact that layer-by-layer deposition can be carried out in mild conditions of pH and temperature, and in aqueous solutions. Furthermore, it does not require the intervention of potentially damaging or denaturing chemical reagents, which makes this technique ideal for the study of sensitive molecules like biopolymers or proteins.

Layer-by-layer assembly on a substrate relies on the alternate adsorption of polyanions and polycations on its surface. Due to the opposing charges, the polyelectrolytes will self-assemble at each step, thus inducing film buildup. This process is schematized in Figure 31. The chemical simplicity of this technique makes it easy to adapt the film's properties through small changes in the experimental setup. Thickness can be adjusted simply by changing the number of (polyanion/polycation) deposition cycles, the pH, or the ionic strength of the solution (Shiratori & Rubner, 2000). It is also easy to change the characteristics of the film by using different polyanion and polycation solutions.

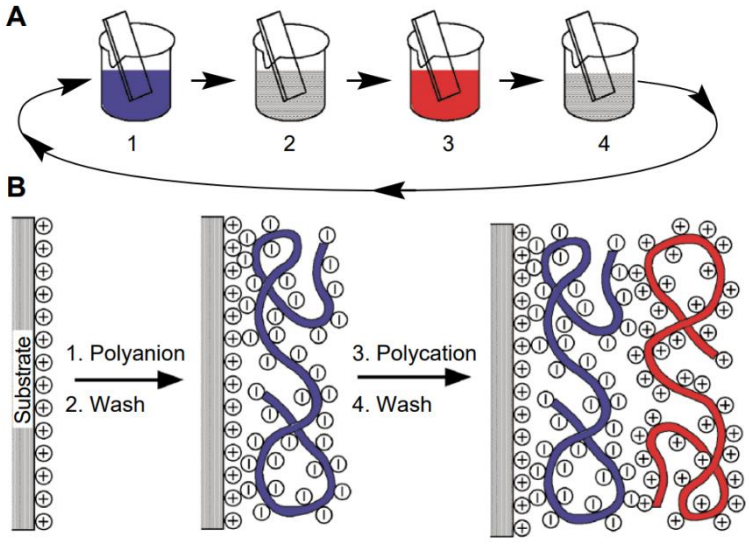


Figure 31: ***Layer-by-layer assembly of PEM films****. (A) Schematic of the film deposition process using slides and beakers. Steps 1 and 3 represent the adsorption of a polyanion and polycation, respectively, and steps 2 and 4 are washing steps. The four steps are the basic buildup sequence for the simplest film architecture, (A/B)n. The construction of more complex film architectures requires only additional beakers and a different deposition sequence. (B) Simplified molecular picture of the first two adsorption steps, depicting film deposition starting with a positively charged substrate. Counterions are omitted for clarity. The polyion conformation and layer*

interpenetration are an idealization of the surface charge reversal with each adsorption step. From Decher, 1997.

Layer-by-layer deposition can be used for polyelectrolytes, but also proteins like collagen (Johansson et al., 2005; Jun Zhang et al., 2005), nanoparticules and nanowires (Srivastava & Kotov, 2008; H. Zhang, Shih, Zhu, & Kotov, 2012), carbon nanotubes (E. Jan & Kotov, 2007), or even living cells (Chetprayoon, Kadowaki, Matsusaki, & Akashi, 2013; Matsusaki, Kadowaki, Nakahara, & Akashi, 2007).

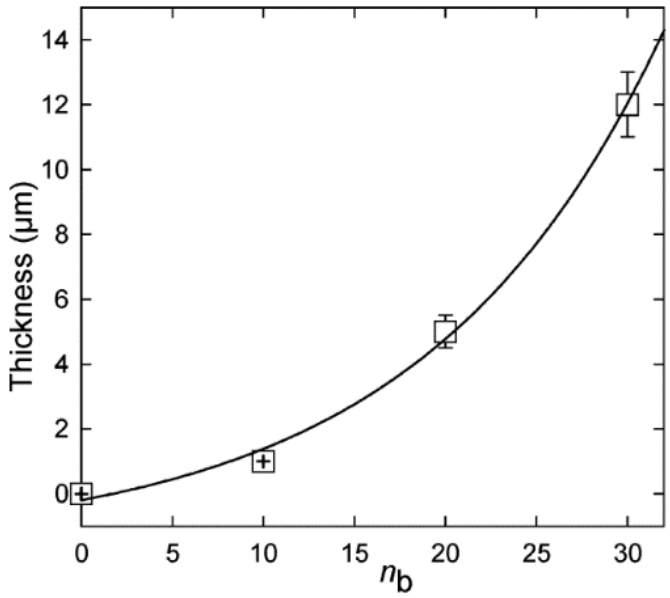


Figure 32: ***Thickness d of the (PLL/HA) films as a function of the number of pairs of layers nb, as measured by AFM (+) and by CSLM (squares) for films built with the automatic dipping machine on 12 mm glass slides****. AFM height measurements were performed by scratching the film (data taken from ref 14), whereas CLSM measurements were performed using PLL-FITC as the last layer to label the whole film (observation of a green band). Error bars represent the uncertainty on the CLSM measurements. From Richert et al. 2004.*

In the works described below, the polycation we used was poly-L-lysine (PLL), and the polyanion was hyaluronic acid (HA). This particular pair of polyelectrolytes grows exponentially, as shown in Figure 32 (Picart et al., 2002; Richert, Engler, Discher, & Picart, 2004). It has also been demonstrated that the mechanical properties of these films can be tuned using carbodiimide chemistry (Boudou, Crouzier, Nicolas, Ren, & Picart, 2011; Richert et al., 2004). Indeed, the use of water soluble carbodiimide (EDC) in combination with N-hydroxysulfo-succinimide induces the crosslinking of the film by linking the carboxylic acid

function of the polyanion to the amine function of the polycation, through an amide bond, as shown in Figure 33.

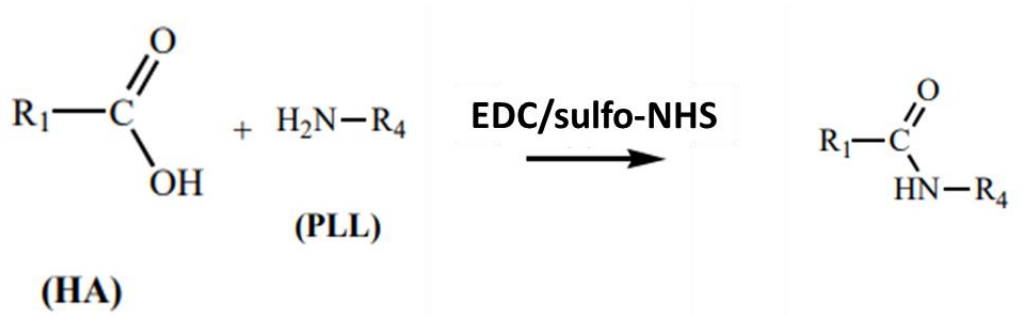


Figure 33: ***Crosslinking of LbL films by means of EDC in combination with sulfo-NHS****. Adapted from Richert et al. 2004.*

The concentration of EDC impacts the mechanical properties of the film, and in particular its Young's modulus, as shown in Figure 34 (Boudou et al., 2011; Francius et al., 2006; Schneider et al., 2006). In the context of the works described below, we used an EDC concentration of 30 mg/mL, which corresponds to a rigidity of approximately 200 kPa. This particular stiffness was chosen for two reasons. First, the Young's modulus of these slightly crosslinked films is close to that of pre-calcified bone, and has been shown to induce osteoblastic differentiation in MSCs (Engler et al., 2006; Halder et al., 2012). Secondly, cells on these slightly crosslinked films have been shown to poorly adhere and spread, which in this case was a desirable property, allowing us to spatially control cell adhesion and spreading, as will be described below (Boudou et al., 2011; Richert et al., 2004).

Of note, the level of crosslinking of the film has also been associated to changes in the internalization of matrix-bound BMP-2 and cell signaling (Gilde et al., 2016).

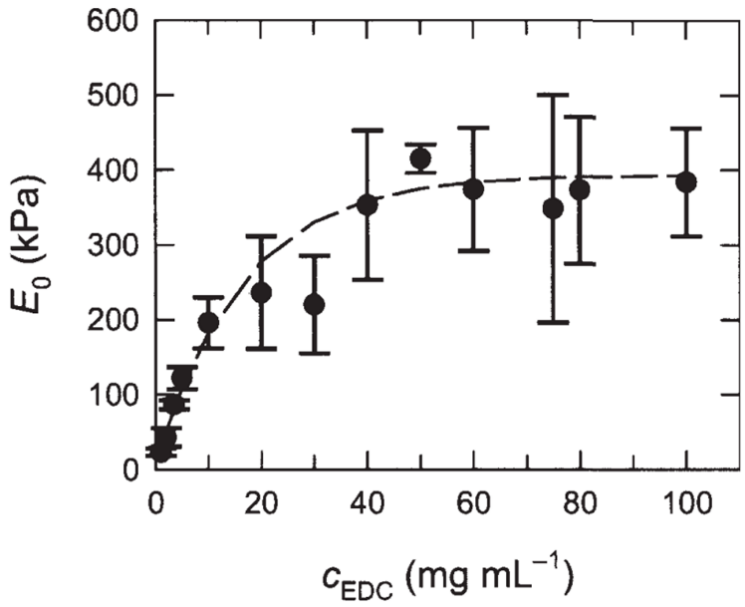


Figure 34: ***Values of the Young's modulus at zero-indentation****. The dashed line is an empirical fit to the data and serves merely to guide the eye. Adapted from Francius et al. 2006.*

As shown in Figure 35, these crosslinked (PLL/HA) films can be loaded with BMP-2 (Crouzier, Ren, Nicolas, Roy, & Picart, 2009), and they have been shown to present a potential for implant coating (Crouzier, Sailhan, et al., 2011; R. Guillot et al., 2016; Raphael Guillot et al., 2013; N. J. Shah et al., 2013; Nisarg J Shah et al., 2014). It has further been demonstrated, using a simple microfluidic device, that these films can be used to spatially control growth factor presentation, and specifically generate gradients of BMP-2 and BMP-7 (Almodóvar et al., 2014).

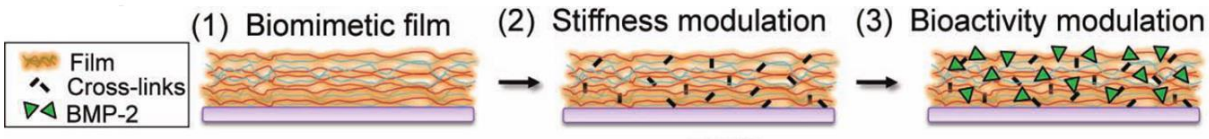


Figure 35: ***Biomimetic thin film combining physical and biochemical cues****. 1) (PLL/HA) film deposition. 2) Film crosslinking allows control over film stiffness. 3) An additional functionality is provided to the film by loading it with the growth factor BMP-2; BMP-2 is trapped within the film and is subsequently delivered to the cells in a "matrix-bound" manner. Adapted from Crouzier et al. 2011.*

On the whole, these results show that (PLL/HA) films can be used as biomimetic matrices, with a stiffness that can be tuned to be close to pre-calcified bone. Furthermore, these films are compatible with BMP-2 presentation and bioactivity. As mentioned above, when slightly crosslinked these soft films do not allow good cell adhesion and spreading, but this can be

rescued by BMP-2. Remarkably, this rescue only occurs when BMP-2 is presented in a matrix-bound manner, and not when it is present in solution (Crouzier, Fourel, Boudou, Albigès-Rizo, & Picart, 2011). This phenomenon is shown in Figure 36. This observation demonstrates that BMP-2 presentation mode is important in the regulation of cell adhesion, and this role suggests that other aspects of cell behavior may also be impacted by BMP-2 presentation mode.

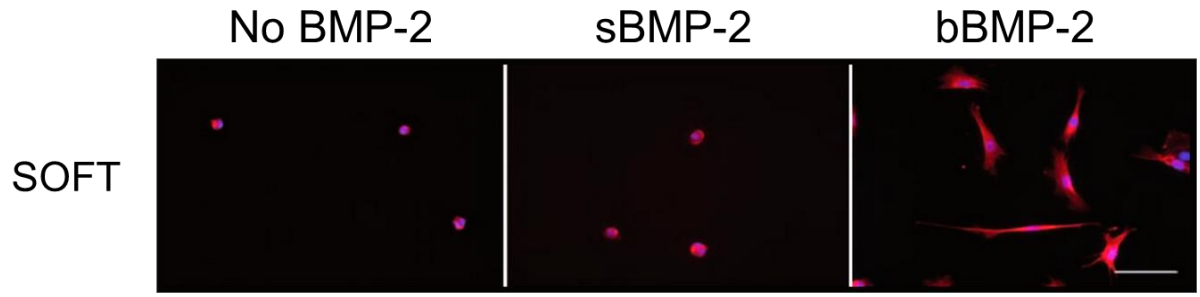


Figure 36: ***Sustained effect of matrix-bound BMP-2 on cell morphology****. Cell morphology is observed 16 hours after plating the cells. (A) Actin and nucleus staining of C2C12 cells revealed that for soft (PLL/HA) films, sBMP-2 did not induce any noticeable effect on cell spreading but bBMP-2 induced a striking increase in cell spreading. The scale bar is 20 µm. Adapted from Crouzier et al. 2011.*

As can be seen in Figure 36, while cells adhere and spread on (PLL/HA) films containing matrix-bound BMP-2, this cell area and shape is uncontrolled. Cells spreading on these biomimetic surfaces will therefore present various shapes and spreading areas, which have been shown to impact cell behavior, and specifically osteoblastic differentiation (McBeath et al., 2004; Y.-K. Wang et al., 2012). Furthermore, since cells are free to adhere anywhere on the film, cell-cell contact is possible, which has also been shown to impact osteoblastic differentiation (Tsai et al., 2012).

These elements mean that in order to specifically decouple the effect of cell spreading on these films from BMP-2 presentation and cell-cell contact, we needed to find a way to control cell adhesion, area and shape. We used two different micropatterning techniques to accomplish this.

4. Micropatterning

Due to their ability to efficiently control many in situ cellular conditions, and in particular cell adhesion, area, and shape, as shown in Figure 37, micropatterning techniques are an extremely useful tool to investigate both cell-cell and cell-ECM interactions. There are several micropatterning techniques, and these can be tailored depending on the specifications of the experiment (Théry, 2010; Wen-Wen, Zhen-Ling, & Xing-Yu, 2009).

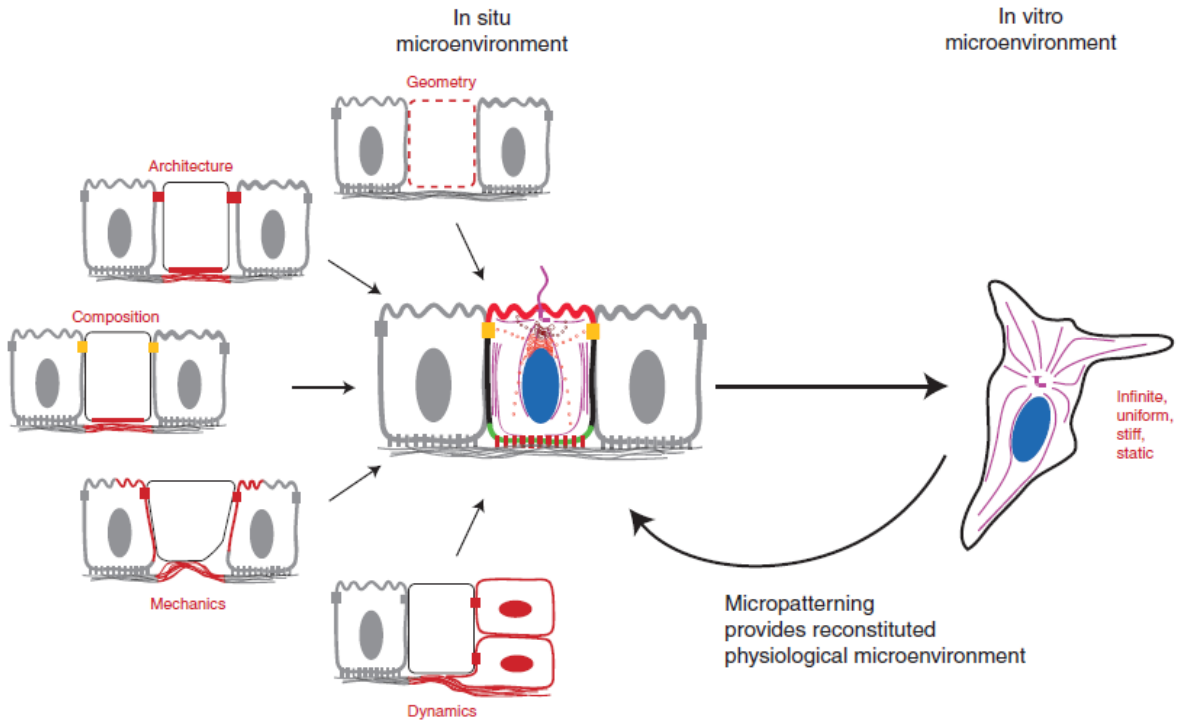


Figure 37: ***The cell microenvironment in situ and in vitro****. In situ (left), the cell microenvironment, i.e. adjacent cells and the ECM (grey), provide a variety of cues (red) for cell morphogenesis, ranging from geometrical constraint to biochemical signaling and mechanical resistance. Under classic culture conditions (in vitro microenvironment), the entirety of this spatial, chemical and mechanical information is lost as the cell microenvironment is a flat plastic or glass surface. Micropatterning methods can be used to modify the microenvironment of cells in culture and restore – to some extent – the external guiding information. These artificial surface treatments render cell culture conditions less artefactual and resemble more closely physiological conditions. From Thery, 2010.*

a. Photolithography

Photolithography consists of using focused light to generate patterned areas on a photosensitive polymer called a photoresist. This light is shone through a mask, which allows the precise control of the patterned areas. Subsequent chemical treatment of the

photoresist is used to reveal these areas (D'Arcangelo & McGuigan, 2015; Gauvin, Parenteau-Bareil, Dokmeci, Merryman, & Khademhosseini, 2012). This process is schematized in Figure 38.

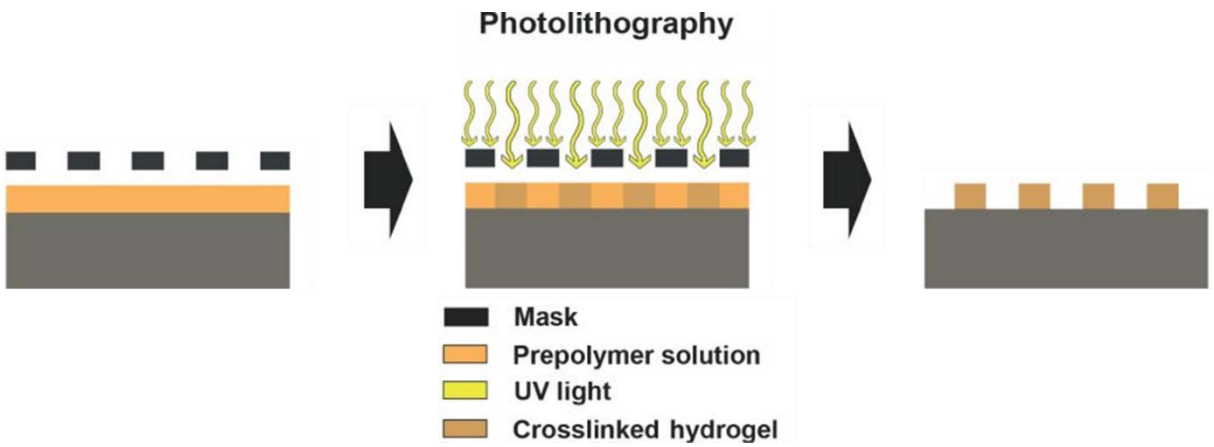


Figure 38: ***Photolithography process****. Adapted from Gauvin et al. 2012.*

The chemicals used for the revelation process are usually strong acids or bases, or organic solvents, and are therefore susceptible to denature proteins (Blawas & Reichert, 1998). Alternative methods using deep-UV have been developed, which provide a way to selectively adsorb fibronectin on patterned areas of a (PLL-g-PEG) coated glass, in non-denaturing conditions (Azioune, Carpi, Tseng, Théry, & Piel, 2010; Azioune, Storch, Bornens, Thery, & Piel, 2009). In the context of this thesis, photolithography was used to generate protein micropatterns on gels of polyacrylamide.

Other teams have also used photoreactive species to pattern proteins using photolithography without the need for chemical treatment (Carrico et al., 2008).

b. Soft lithography

Soft lithography designates several techniques which use polydimethylsiloxane (PDMS) to replicate the structure of a photoresist obtained by photolithography (D. Qin, Xia, & Whitesides, 2010). This avoids direct contact between proteins and the potentially denaturing chemicals used in photoresist revelation. Since PDMS is transparent and does not exhibit any significant autofluorescence, these samples can be observed directly by light and

fluorescent microscopy. Soft lithography has been used for microcontact printing, microfluidic patterning and stencil patterning.

i. Microcontact printing

Microcontact printing is frequently used for protein micropatterning, due to its low cost, facility to implement and submicron resolution.

This technique consists of using PDMS to generate a stamp, which is then used to transfer the protein of interest onto the substrate (Hauff et al., 2015; K. Shen, Qi, & Kam, 2008; J. L. Tan, Tien, & Chen, 2002; Théry & Piel, 2009). Depending on the substrate used, two separate methods can be used: the casting method or the lift-off method (von Philipsborn et al., 2006), as shown in Figure 39. In the works described below, microcontact printing with the casting method was used for all experiments on (PLL/HA) films.

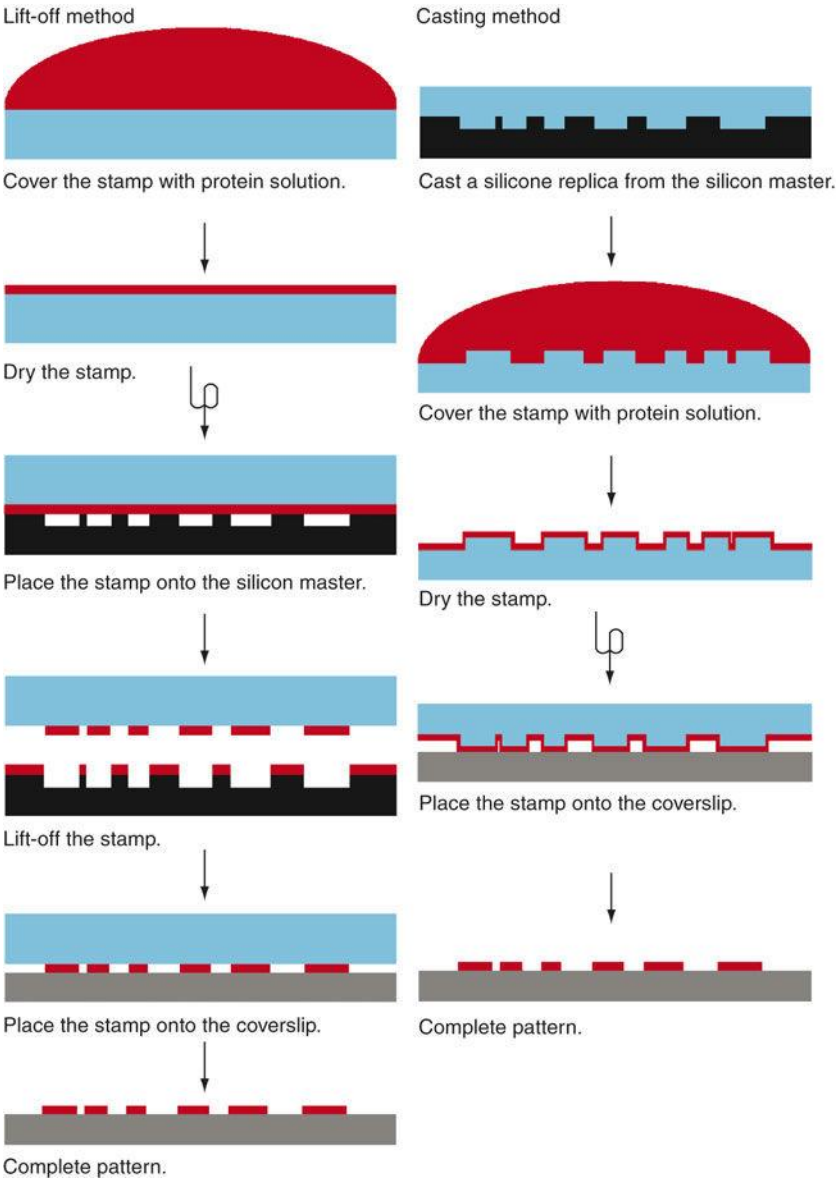


Figure 39: ***Two different methods to generate a protein pattern (red) onto a coverslip (gray) from a silicon master (black) using a stamp (blue) made from PDMS elastomer****. From von Philipsborn et al., 2006.*

We carried out our experiments using manual microcontact printing. In recent years, however, automatized printing robots like the Innostamp by Innopsys have been commercialized to facilitate this process. These present the advantage of increasing the reproducibility of the printing process, removing user-dependency, and allowing the precise control of additional parameters such as temperature and humidity.

ii. Microfluidic patterning

Microfluidic patterning is carried out using a process which is very similar to microcontact printing. For microfluidic patterning, however, when the PDMS mold is placed on the substrate, it forms a networks of microchannels, in which a solution containing the protein of interest is introduced, leading to the adsorbtion of the protein in the channels. This process is schematized in Figure 40 (Kim, Xia, & Whitesides, 1995; W. Tan & Desai, 2003). For example, microfluidic patterning has been used for the fabrication of contractile cardiac organoids (Khademhosseini et al., 2007), or to generate BMP-2 gradients on PEM films (Almodóvar et al., 2014).

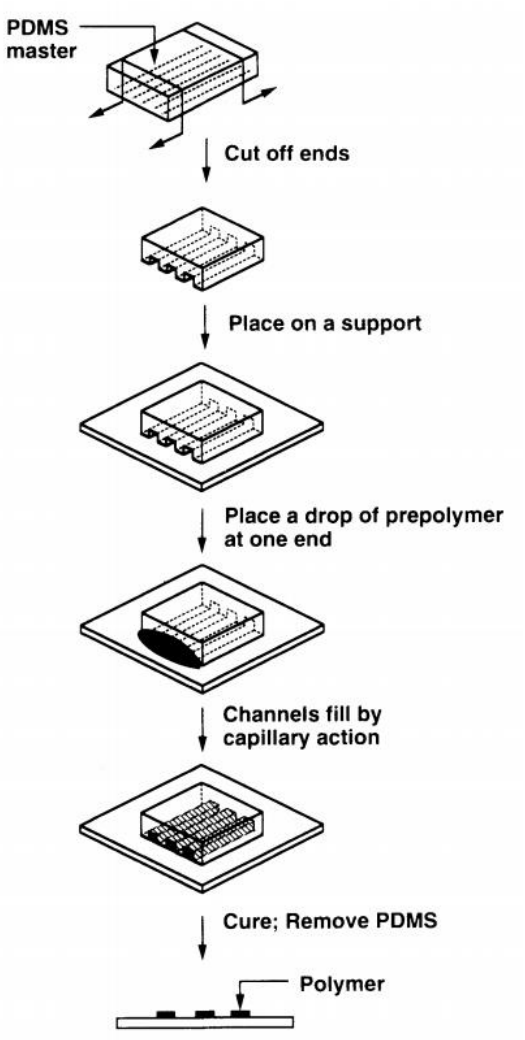


Figure 40: ***Schematic diagram of the microfluidic patterning technique****. From Kim et al. 1995.*

iii. Stencil patterning

Stencils consist of thin sheets of PDMS, containing holes in the areas that need to be patterned. These sheets are obtained in a manner quite similar to the stamps used for microcontact printing. They are then placed on the substrate, and the solution containing the protein of interest is placed on the stencil-covered substrate and left to adsorb. Once the stencil is peeled off, only the areas which were not covered by the stencil present protein, as shown in Figure 41 (Du, Ding, Liu, Wathuthanthri, & Choi, 2017; Folch, Jo, Hurtado, Beebe, & Toner, 2000; Masters et al., 2012).

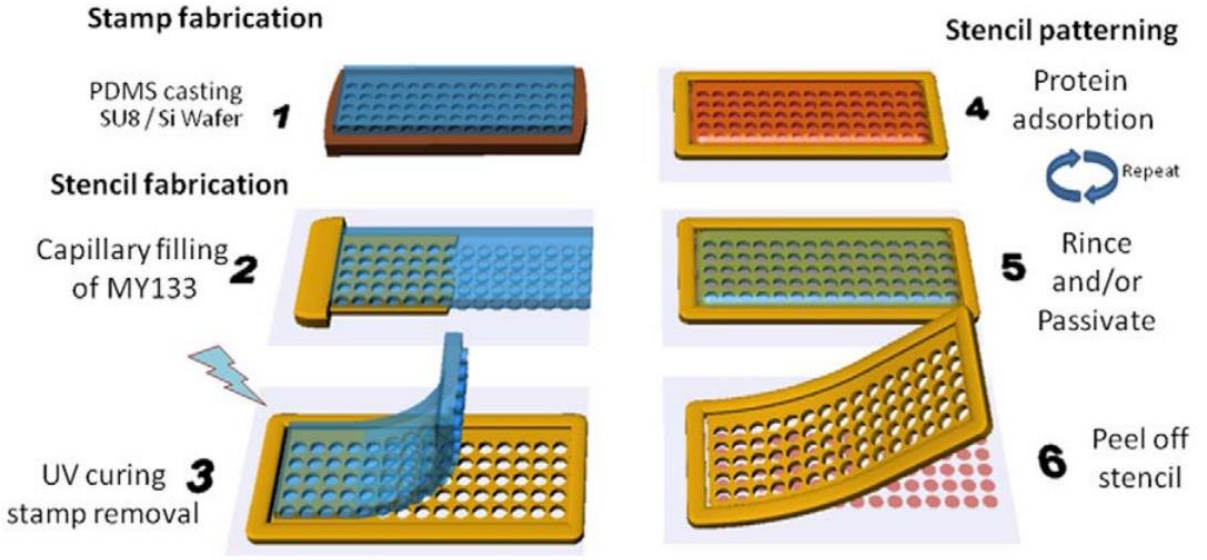


Figure 41: ***Fabrication steps for the UV curable polymer stencil membrane****. A negative PDMS stamp is first fabricated using standard SU8 lithography. The stamp is then placed onto a flat substrate (e.g. glass, PDMS, Petri dish). UV curable monomers are introduced by capillary flow into the gap between the stamp and the substrate. After UV curing the stamp is removed. The substrate is left for incubation with a dilute suspension of the protein of interest. Successive cycles of wash/passivation/incubation steps are then performed depending on the number of protein to be adsorbed on the patterns. After final washing the stencil is removed. Further incubation with an antifouling agent or another protein can then be performed. Notice that the proteins stay hydrated throughout the process. From Masters et al. 2012.*

c. Other techniques

There are other techniques, known as direct write techniques, which can be used to micropattern proteins. These techniques present interesting feature and uses, but are not yet as commonly used as the patterning methods described above.

One of these methods is laser-based patterning, a non-contact form of patterning in which a laser is used to guide and deposit proteins on the substrate (Barron et al., 2004; Tasoglu & Demirci, 2013), or selectively remove them (Heinz, Hoh, & Hoh, 2011).

Inkjet printing, another form of non-contact printing, is a techniques which relies on dropping proteins solutions – notably ECM proteins – (Calvert, 2001; Delaney, Smith, & Schubert, 2009) or cell suspensions (Roth et al., 2004; T. Xu, Jin, Gregory, Hickman, & Boland, 2005) on the substrate.

Finally, dip-pen lithography is a micropatterning technique using an atomic force microscope (AFM) tip to transfer a protein solution onto the substrate using capillary forces, as shown in Figure 42.

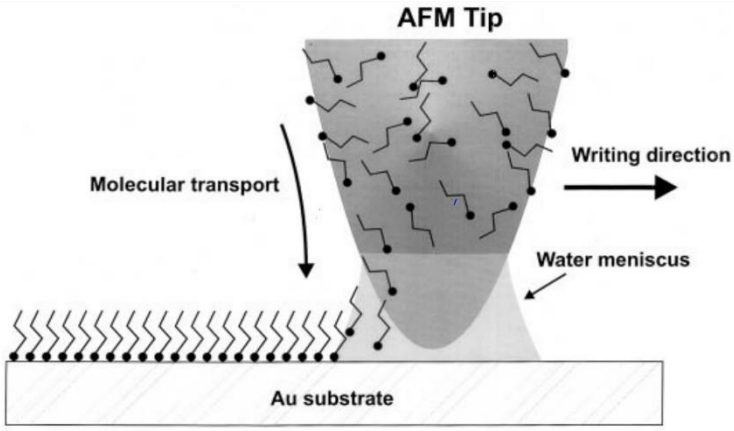


Figure 42: ***Schematic representation of dip-pen nanolithography*** *(DPN). A water meniscus forms between the AFM tip coated with ink and the Au substrate. The size of the meniscus, which is controlled by relative humidity, affects the transport rate, the effective tip-substrate contact area, and DPN resolution. From Piner et al. 2014.*

On the whole, the patterning techniques described above – in particular photolithography and microcontact printing – can be combined with engineered ECMs, such as PAA gels and PEM films. In doing so, we are able to benefit from the properties of the engineered ECM in terms of integrin recruitment, growth factor presentation and mechanical signals, and combine them with the spatial control which is made possible by micropatterning techniques.

OBJECTIVES OF THE THESIS

Overall, the elements described above paint a picture of the stem cell niche, the factors regulating it, and the tools that can be used to study these factors, through the use of engineered matrices and spatial patterning techniques which allow the spatial control of cell spreading.

Specifically, we have seen that **the stem cell niche is a tightly regulated microenvironment that controls stem cell self-renewal, proliferation and differentiation.** These cell behaviors are dictated by cell-cell interactions, secreted factors, and cues provided by the ECM. These cues can be biochemical, as demonstrated by the ability of ECM proteins to retain growth factors, and serve as a reservoir for growth factor presentation. They can also be mechanical, with factors like stiffness affecting cell shape and differentiation. Many of these mechanisms are mediated by integrins, transmembrane receptors that sense the local environment and interact with other receptors like such as growth factor receptors, which are present at the cell surface and regulate downstream signaling.

When it comes to the MSC niche and osteoblastic differentiation, the literature strongly suggests a role of growth factor BMP-2 in this regulation, through Runx2 activation. Furthermore, studies have shown that high matrix rigidities can induce osteoblastic differentiation, even in the absence of biochemical cues. Finally, it has been shown that cell shape and spreading can modulate BMP-2 induced signaling.

The ability of BMP-2 to induce bone formation and osteogenic-related signaling pathways makes it an interesting candidate to study osteoblastic differentiation. A further advantage of using BMP-2 when studying the cell's microenvironment is its ability to bind to ECM proteins, and in particular fibronectin, thus making it possible to present BMP-2 in a matrix-bound manner.

Several studies have already examined matrix-bound presentation of BMP-2. Indeed, BMP-2 immobilized either by physical adsorption (Crouzier, Fourel, et al., 2011; Fourel et al., 2016) or by covalent grafting (Lagunas et al., 2013; Wei, Pohl, Seckinger, Spatz, & Cavalcanti-Adam, 2015) have been shown to regulate cell behavior quite distinctly from BMP-2 in solution.

This effect is currently poorly known and is likely due to the close proximity and crosstalk of integrin-binding domains of FN and BMP-2 (Fourel et al., 2016; F. Lin et al., 2011; Taipale & Keski-Oja, 1997; Wei et al., 2015). It has indeed been shown that the presence of FN in the pericellular coat is necessary for BMP-2-mediated signaling (Fourel et al., 2016).

A consequence of this association of BMP-2 with ECM proteins in vivo is that the **spatially patterned presentation of BMPs by the ECM balances progenitor cell renewal and differentiation in the stem cell niche** (Brown, Muthukrishnan, & Oxburgh, 2015; Crisan et al., 2015; Khurana et al., 2014; Kosinski et al., 2007) and guides numerous developmental and repair steps (Bénazet & Zeller, 2009; Genikhovich et al., 2015; Künnapuu et al., 2014).

These elements highlight the need to develop simple methods to recreate a niche microenvironment in vitro. Indeed, methods presenting specific mechanical properties, ECM proteins and locally immobilized GFs are important for elucidating fundamental features of developmental biology, as well as being a possible solution to guide and enhance tissue engineering approaches.

Many ECM proteins have been patterned by microcontact printing on stiff substrates, aimed to study cell adhesion, migration, division and differentiation (C. S. Chen, Mrksich, Huang, Whitesides, & Ingber, 1997; McBeath et al., 2004; Mrksich & Whitesides, 2003; Théry et al., 2005). Wang *et al.* thus used FN micropatterns to demonstrate that the differentiation of human MSCs into osteoblasts in response to BMP-2 added in solution in the culture medium (i.e. soluble BMP-2) actually depends on cell shape and cytoskeletal tension (Y.-K. Wang et al., 2012).

In contrast, very few techniques exist for patterning GFs such as BMP-2 without chemically modifying the protein (Migliorini, Valat, Picart, & Cavalcanti-Adam, 2016). Recently, Cavalcanti-Adam *et al.* developed new approaches for immobilizing BMP-2, by using biotinylated BMP-2 or a heterobifunctional linker to covalently graft BMP-2 to a glass coverslip (Hauff et al., 2015; Schwab et al., 2015). This team was able to demonstrate that immobilized BMP-2 triggered early differentiation signaling and increased migration of C2C12 myoblasts. Likewise, Martinez *et al.* used biotinylation to generate patterns with a

continuous gradient of BMP-2 on poly(methyl methacrylate) substrates (Lagunas et al., 2013), and demonstrated the relevance of this technique for the in vitro screening of the early stages of ostegenesis in C2C12 cells. While techniques relying on the covalent binding of BMP-2 on a substrate are a useful tool, chemical modifications of BMP-2 may hinder the biological activity of the BMP-2 or affect cell behaviors, like for example internalization (Alborzinia et al., 2013; Y. Ito, Chen, & Imanishi, 1998; Nakaji-Hirabayashi, Kato, Arima, & Iwata, 2007).

Currently, the only existing methods for creating patterns of unmodified, matrix-bound BMP-2 on ECM coatings are based on inkjet printing. Campbell *et al.* thus used the natural affinity between BMP-2 and fibrin to generate 0.5-1 mm^2 squares of immobilized BMP-2 on a fibrin substrate (Ker et al., 2011; Phillippi et al., 2008). They demonstrated the possibility to guide a stem cell population toward multiple fates on a single substrate and in a spatially defined manner. But the millimetric resolution of this approach limits its use to large, multi-cellular patterns, on which a part of the cell population responds to BMP-2 patterns differently than the rest of the population outside the patterns, independently of cell adhesion.

We previously reported that soft biopolymeric films composed of poly(L-lysine) (PLL) and hyaluronic acid (HA) can retain high and tunable quantities of BMP-2 (Crouzier et al., 2009). This interesting property was used to create large, centimeter-scale gradients of matrix-bound BMP-2 by microfluidics (Almodóvar et al., 2014). Moreover, the presentation of matrix-bound BMP-2 rescued C2C12 myoblast adhesion on these otherwise non-adhesive soft biopolymeric films (Crouzier, Fourel, et al., 2011) via a combined action between BMP receptors and integrin receptors (Fourel et al., 2016).

While these works allow a control of matrix stiffness (through film crosslinking) and matrix-bound presentation of BMP-2, they do not allow a precise control of cell spreading. Keeping in mind that cell spreading has been linked to cytoskeletal tension, and that mechanical forces have been shown to impact cell behavior, there is a need to normalize cell size and shape in order to investigate the role that cell spreading plays on the BMP-2-induced signaling. Furthermore, as described above, cell-cell interactions play an important role in

regulating the stem cell niche. On biopolymeric films presenting matrix-bound BMP-2, the lack of spatial control in the presentation of BMP-2 makes it extremely difficult to avoid cell-cell contact on these substrates.

In the works described below, our goal was to build on these results to generate cellular- and subcellular-sized patterns of unmodified BMP-2, within and without a FN matrix, and on soft biopolymeric films. We wished to use these patterns to examine the influence of the size of these BMP-2 micropatterns on cell spreading, cytoskeleton organization and early osteogenic trans-differentiation signaling, with single-cell precision. Finally, we wanted to investigate how intracellular forces impacted BMP-2-activated signaling pathways, and how the cell was able to interact with the ECM through bidirectional signaling.

MATERIALS AND METHODS

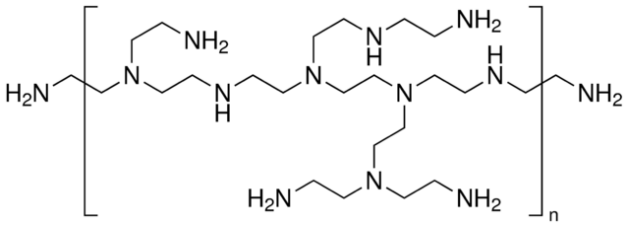
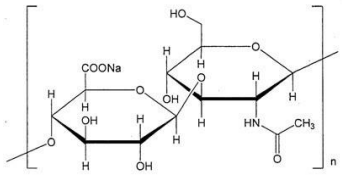
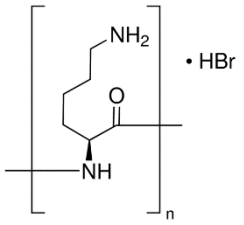
I. Micropatterning

1. Microcontact printing on PEM films

a. Polyelectrolyte solutions

Polyelectrolyte multilayer (PEM) films were constructed using the layer-by-layer deposition of polyanions and polycations on a substrate. Three polyelectrolyte solutions were used for the film buildup:

- PEI: Poly(ethyleneimine) (181978, *Sigma-Aldrich*)
- HA: Sodium hyaluronate (HA500K-5, *Lifecore Biomedical*)
- PLL: Poly(L-lysine) hydrobromide (P2636, *Sigma-Aldrich*)

Polyelectrolyte	**Chemical structure**	**Average molecular weight**	**Charge at pH 7.4**
Poly(ethyleneimine)		750 kDa	+
Sodium hyaluronate		301-450 kDa	-
Poly(L-lysine) hydrobromide		30-70 kDa	+

All polyelectrolytes were dissolved in a Hepes-NaCl buffer:

- 20 mM N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid (Hepes)

- 0.15 M sodium chloride (NaCl)
- Adjusted to pH 7.4 using potassium hydroxide (KOH)
- Filtered at 0.22 µm (SLGS033SB, *Merck*)

PEI, HA and PLL were dissolved in this Hepes-NaCl buffer at 2, 1 and 0.5 $mg.mL^{-1}$, respectively.

PEI and HA are slow to dissolve, and were therefore prepared in advance and left to dissolve overnight at 4 °C. PLL was dissolved approximately 20 to 30 minutes before use.

The rinsing steps were carried out between each incubation in the polyelectrolyte solutions, in a solution of 0.15 M NaCl, adjusted to pH 6.4 using sodium hydroxide (NaOH).

b. Film buildup

PEM film deposition was carried out on 14 mm diameter glass coverslips. Prior to film deposition, the coverslips were cleaned in Hellmanex III (320003, *Hellma-France*) 0.1 % at 50° C for 15 minutes, then rinsed abundantly with MilliQ water to remove any trace of Hellmanex III, and dried using a flow of sterile nitrogen.

A first bilayer of PEI/HA was deposited manually on the substrates for better anchoring of the PEM film to the underlying substrate.

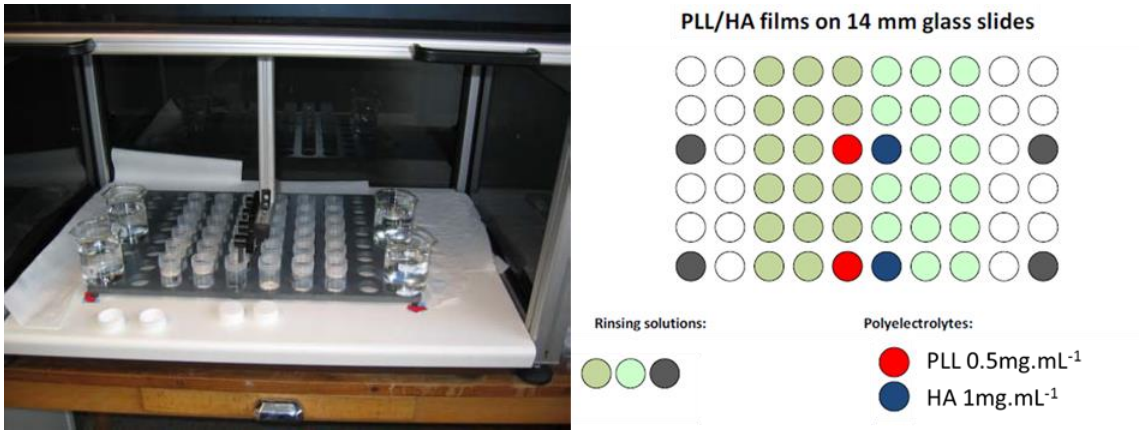
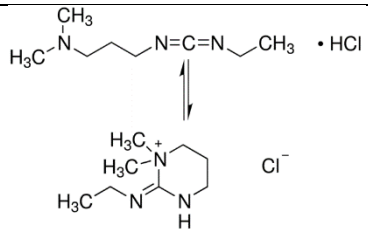
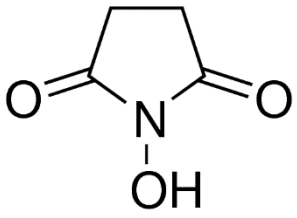


Figure 43 : ***Layer-by-layer film deposition****. Automatic dipping robot (left) and scheme of polyelectrolyte and rinsing solution placement on the rack (right).*

Subsequently, the buildup of the film made of 24 (PLL/HA) bilayers ($(PLL/HA)_{24}$) was done with an automatic dipping machine (Dipping Robot DR3, *Kierstein GmbH*), using alternate steps of incubation in the polyelectrolyte solutions, and rinsing.

c. Crosslinking

The subsequent crosslinking of the PEM films was achieved using 1-Ethyl-3-(3-Dimethylamino-propyl) carbodiimide (EDC; E7750, *Sigma-Aldrich*) at 30 $mg.mL^{-1}$ and N-hydrosulfosuccinimide (sulfo-NHS, *Sigma-Aldrich*) at 11 $mg.mL^{-1,}$, as previously described (Schneider et al., 2006). Films were placed in this solution for 18 hours at 4 °C. The crosslinking reaction was then stopped through extensive rinsing steps using the Hepes-NaCl buffer.

1-Ethyl-3-(3-Dimethylamino-propyl) carbodiimide	
N-hydrosulfosuccinimide	

d. Sterilization

For experiments requiring cell seeding on the PEM films, an additional sterilization step was carried out post-crosslinking. To this end films, still in the Hepes-NaCl buffer, were placed in

the cell culture hood under UV light for 20 minutes. The $(PLL/HA)_{24}$-coated coverslips were then ready for cell seeding.

If cell seeding was not carried out immediately after sterilization, the films were further rinsed in sterile MilliQ water to remove any NaCl present in the film, and dried under the cell culture hood before being stored at 4 °C until needed.

e. Microcontact printing

i. "Ink" preparation

Prior to the printing process, « ink » solutions containing the proteins of interest (namely BMP-2 and Fibronectin, FN) were prepared.

rhBMP-2 (Clinical Grade, *Wyeth BioPharma*) aliquots were prepared using a clinically-approved kit, containing 12 mg of rhBMP-2 in 300 mg of powder. This powder is dissolved in a sterile solution of 1 mM HCl, and the rhBMP-2 concentration is checked using absorbance spectrometry at 280 nm with a NanoDrop 2000 spectrophotometer (*Thermo Scientific*), with $\varepsilon_{BMP\text{-}2(280\ nm)}$ = 39 500 $M.cm^{-1}$. The rhBMP-2 solution is then aliquoted and stored at -20 °C until used.

Fibronectin (11080938001, *Sigma-Aldrich*) was aliquoted at 1 $mg.mL^{-1}$ in PBS.

ii. Pattern visualization

1. rhBMP-2

For visualization purposes, part of the rhBMP-2 used in the printing process was labeled with a 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (CF; *Boehringer*, Mannheim), as previously described (Crouzier et al., 2009).

For the labeling process, the pH of the rhBMP-2 solution was increased to 7.5 using a solution of 50 mM sodium bicarbonate pH 8.4, to allow the grafting of the dye to the BMP-2 (NHS ester chemistry). 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester was then added to the solution, with a molecular ratio of 20:1 (dye:BMP-2), and left to incubate under slow agitation for 2 h at room temperature, and protected from the light.

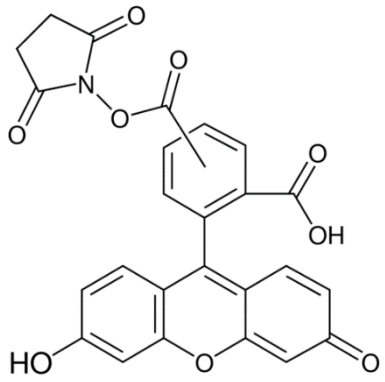


Figure 44: ***Molecular structure of 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester.***

The solution was then reacidified to pH 4 using a 0.2 M acetate buffer pH 3, before purification. Purification was carried out by size-exclusion chromatography on G-25 Sephadex columns (*General Electric Healthcare*). The fractions collected were analyzed by absorbance spectrometry with the NanoDrop 2000 spectrophotometer to determine BMP-2 concentration and the grafting ratio of the dye. In the results detailed below, 36 % of the BMP-2 was labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester. These fractions were then stored at -20 °C until needed.

2. Fibronectin

For the conditions containing fibronectin and no BMP-2 in the pattern (later referred to as FN alone), the inks were comprised of 45 $\mu g.mL^{-1}$ of FN and 5 $\mu g.mL^{-1}$ of Alexa Fluor 488-

labeled fibrinogen from human plasma (Fibrinogen^{A488}, *Life Technologies*), which allowed visualization of the patterns on the PEM film using confocal microscopy.

iii. Master preparation

Silicon masters were fabricated by deep UV photolithography of a positive resist designed for high aspect ratios (AZ TX 1311, *Microchemicals*).

The resist was first spread on a silicon wafer for 2 seconds at 1500 rpm (acceleration 1000 rpm/s), then spun for 20 seconds at 800 rpm. The deposited resist was soft baked for 2 minutes at 70 °C, then 2 minutes at 140 °C before cooling down to room temperature. The resist was insolated (40 mJ/cm²) under vacuum contact before a post-exposure bake for 2 minutes at 60 °C, 2 minutes at 105 °C, and 5 minutes to cool down to room temperature. The exposed resist was finally developed for 3 minutes in MF-26A (*Microposit*) on a shaker and rinsed thoroughly in deionized H2O.

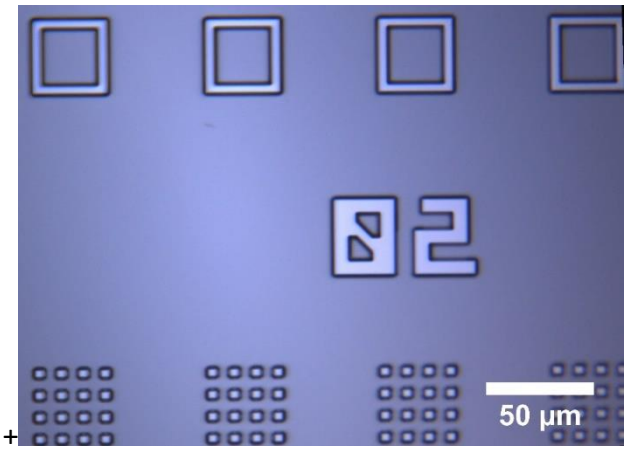


Figure 45: ***Representative image of patterns on a silicon master****.*

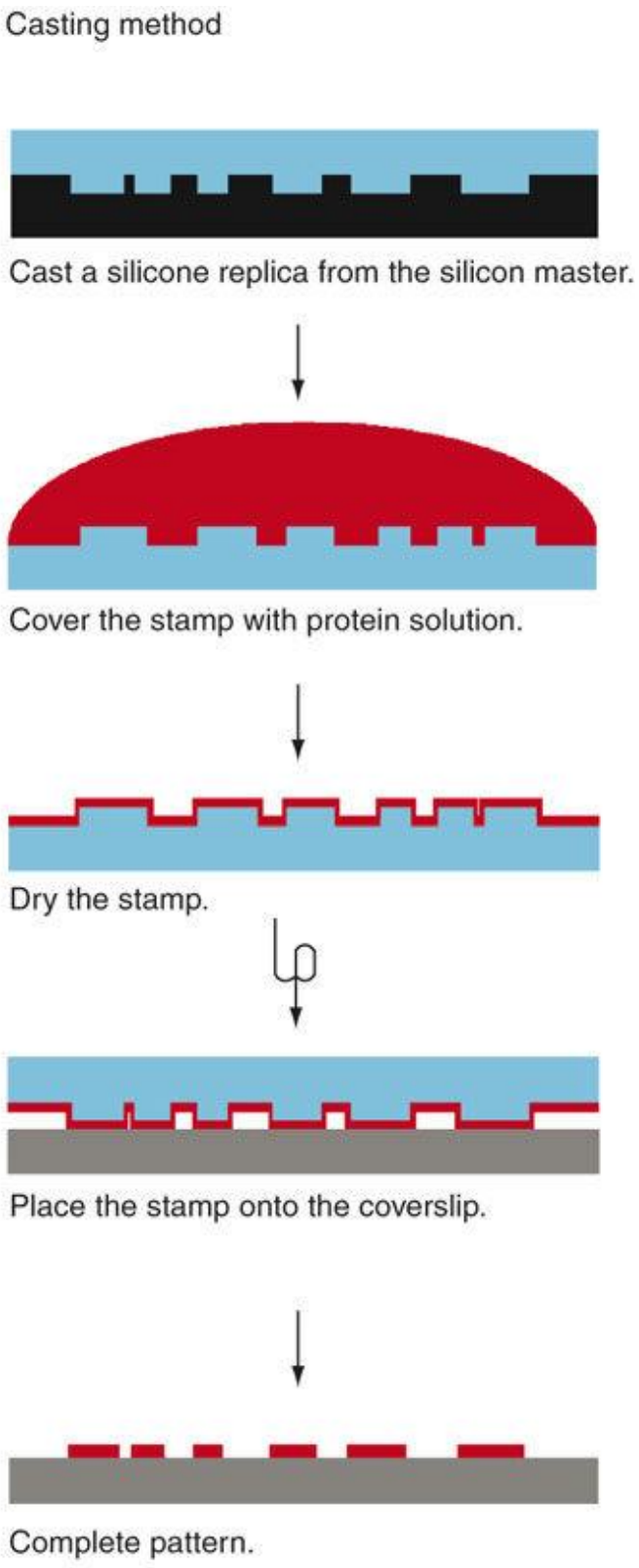
iv. Stamp preparation

Polydimethylsiloxane (PDMS) stamps were made by casting Sylgard 184 (*Dow Corning*) liquid prepolymer over the silicon master.

Before the first replication, and to facilitate the removal of the PDMS from the surface of the wafer during the replication process, this surface was activated using oxygen plasma (Evactron C De-Contaminator, *XEI Scientific*), then silanized overnight with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (448931, *Sigma-Aldrich*) under a vacuum hood. The PDMS mixture was prepared by combining the liquid prepolymer and curing agent at a 10:1 ratio, mixing vigorously for several minutes, and degassing the mixture to remove the bubbles which had formed during the mixing step. The PDMS mixture was then poured over the silicon master, spin-coated for 60 seconds at 250 rpm, with an acceleration of 100 rpm/s, to insure that the entire surface of the silicon wafer was covered by a flat and homogeneous layer, and cured at 100 °C for 15 minutes.

Upon curing, the elastomeric stamps were peeled off, washed with ethanol, and dried under a flow of sterile nitrogen. They were then stored at room temperature in a dust-free environment until needed.

1. Printing process



Stamps were then coated with the prepared ink solutions, containing either FN, BMP-2, or a mixture of both at equal concentrations. PDMS being highly hydrophobic, 100 µL were placed on the surface of the PDMS stamp, flattened with a glass coverslip in order to coat the entire surface of the stamp, and left to incubate for 1 hour.

After rinsing with Dulbecco's phosphate buffered saline (PBS; 14190, *Gibco*) to remove any unattached protein, the inked stamps were blown dry under sterile nitrogen flow. Removing any drops of liquid from the surface of the stamp is important in order to avoid capillarity during the printing process, which results in an unfaithful reproduction of the stamp.

*Figure 46: **Microcontact printing process**. Adapted from Philipsborn et al. 2006.*

Simultaneously, the crosslinked $(PLL/HA)_{24}$ films were placed in MilliQ water for 30 minutes to remove any trace of NaCl. They were then gently blow-dried under sterile nitrogen flow and placed in conformal contact with an inked PDMS stamp for 1 minute, as shown in Figure 46.

Once printed, and before cell deposition, films were thoroughly rinsed with the Hepes-NaCl buffer, to remove any poorly attached protein.

2. Microcontact printing on PDMS

Glass coverslips were treated with oxygen plasma for 2 minutes. PDMS was then deposited on the coverslip, which was then spin-coated at 500 rpm for 10 seconds, then 4000 rpm for 10 seconds, with an acceleration of 1000 rpm/s. The PDMS-coated coverslip was then cured on a hotplate at 110° for 10 minutes. The substrates were protected at all times to avoid dust deposition on the PDMS.

The printing process then took place, as described above.

3. Deep UV micropatterning on polyacrylamide gels

Regardless of the method of photopatterning used, the photomask is thoroughly cleaned prior to the photopatterning step. This is carried out through successive rinses in MilliQ water and isopropanol, followed by oxygen plasma treatment. Methods for photopatterning were carried out as previously described by Vignaud *et al* (Vignaud et al., 2014).

Briefly, glass coverslips were activated using oxygen plasma, and then left to incubate on a drop of pLL-PEG solution (25 µL/cm², *Susos*) on parafilm for 30 minutes. Once passivated with pLL-PEG, the coverslips were treated with UV light (deep UV insolation), shone through the previously described chrome photomask. This allows us to burn the layer of pLL-PEG only at desired positions, which will then be left open for protein adsorption.

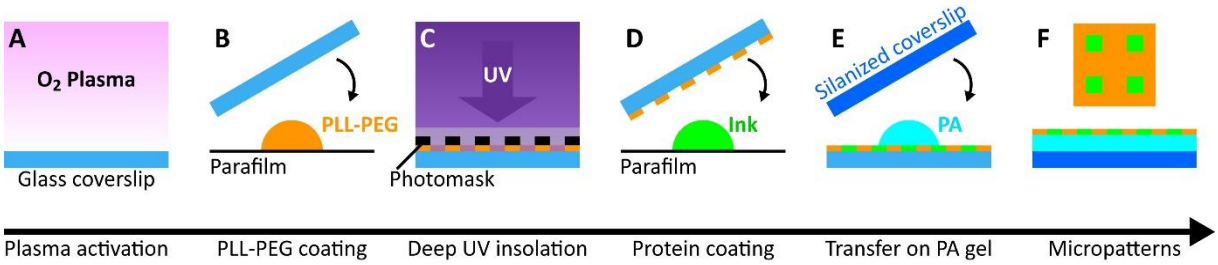


Figure 47: ***Transfer on a polyacrylamide (PAA) gel from a micropatterned glass coverslip*** *(referred to as the "glass method"). This process consists in glass activation by plasma (A), coating with the repellent compound poly-L-lysine–PEG (pLL–PEG) (B), surface activation of the surface through a chrome photomask using deep UV (C), extracellular matrix (ECM) protein adsorption on the UV-activated sites (D) leading to the production of a glass micropatterned coverslip as previously described (Azioune et al., 2010). Then, a drop of PAA solution mix is sandwiched between the patterned coverslip and a silanized glass coverslip (E). After 30 min polymerization, the patterned coverslip is detached from the acrylamide gel while ECM protein remains on the gel (F). Note that due to the diffraction of the UV light at step (C), the shape of the final micropatterns is larger than the original on the photomask.*

The ink solutions (25 µL/cm²) were then left to incubate under these insolated coverslips, to allow protein adsorption. Finally, the acrylamide gel was polymerized between the patterned coverslip and a silanized coverslip. During this step, the protein is transferred to the surface of the polyacrylamide gel, and upon detachment the gel remains attached to the silanized coverslip, thus resulting in an acrylamide surface which is micropatterned with the protein of interest.

The concentrations of acrylamide and bisacrylamide (*Sigma-Aldrich*) used in the traction force microscopy (TFM) experiments carried out below were chosen in order to obtain a gel stiffness of 14 kPa.

II. Cell culture and seeding conditions

1. Cell types

a. C2C12 myoblasts

Murine C2C12 skeletal myoblasts (< 15 passages; CRL-1772, *American Type Culture Collection* (*ATCC)*) were cultured in a 1:1 Dulbecco's Modified Eagle Medium (DMEM):Ham's F12 medium (11320, *Gibco*) supplemented with 10 % fetal bovine serum (FBS, *PAA laboratories*).

b. D1 mesenchymal stem cells

Murine D1 MSCs (< 15 passages; CRL-12424, *ATCC*) were cultured in Minimum Essential Medium Eagle Alpha Modification medium (α-MEM; M4526,*Sigma Aldrich*) supplemented with 10 % FBS.

c. Immortalized human myoblasts

Human myoblasts (hMyo) were immortalized from a clone of primary human muscle stem cells isolated from a 53-year-old male individual, provided by the Institute of Myology (Paris) and cultured in a growth medium containing DMEM:Medium 199 (4:1) supplemented by 25 $\mu g.mL^{-1}$ fetuin (10344026, *Gibco*), 5 $\mu g.mL^{-1}$ insulin (I2643, *Sigma-Aldrich*), 0.2 $\mu g.mL^{-1}$ dexamethasone (D1756, *Sigma-Aldrich*), 0.5 $ng.mL^{-1}$ basic Fibroblast Growth Factor (FGF),

5 ng.mL^{-1} human Epidermal Growth Factor (EGF), 20 % FBS. All culture media were supplemented with 100 U mL^{-1} penicillin G and 100 µg.mL^{-1} streptomycin (15140, *Gibco*).

d. Drugs

For experiments using pharmacological inhibitors, Myosin II inhibitor blebbistatin (*Calbiochem*), LIM kinase inhibitor Pyr1 (kindly provided by Laurence Lafanechère, IAB, Grenoble) and ROCK inhibitor Y-27632 (*Calbiochem*) were introduced in the growth medium at a 20 µM, 25 µM and 10 µM concentration, respectively.

The solvent used for Y-27632 was water, and for blebbistatin and Pyr1 was dimenthyl sulfoxide (DMSO). An additional control condition containing DMSO was therefore carried out to rule out an effect of DMSO on cell behavior in the presence of these particular pharmacological inhibitors.

e. Seeding conditions

Cells were deposited on the printed $(PLL/HA)_{24}$ films at a density of 10^4 cells/cm^2. The substrates were then placed in an incubator at 37 °C, 5 % CO2 for the duration of the desired experiment.

f. siRNA interference

Cells were transfected with small interfering RNA (siRNA) against LIMK1&2 or ROCK1&2 (ON-TARGET plus SMARTpool) as previously described (Fourel et al., 2016). Briefly, cells were incubated with a transfection mix of Lipofectamine RNAiMAX Reagent (*Gibco*) and siRNA in

Opti-MEM medium (*Gibco*) for two consecutive 24-hour periods before seeding on patterned PEM films.

For experiments with integrin silencing, cells were transfected with siRNA against β_1, β_3 or β5 integrins (ON-TARGET plus SMARTpool). A scrambled siRNA (all stars negative control siRNA, *Qiogen*) was taken as control.

III. Immunofluorescent staining

After being cultured on the micropatterns, cells were rinsed with PBS, then fixed with 3.7 % formaldehyde in PBS for 20 minutes at room temperature. After fixation, cells were once again rinsed with PBS, before being permeabilized with 0.2 % Triton X-100 in TBS [50 mM Tris-HCl, 0.15 M NaCl, pH 7.4] and blocked with 2% Bovine Serum Albumine (BSA) (*Aurion*) in TBS. The samples were then incubated with primary antibodies against p-SMAD1/5/8 (13820, *Cell Signaling*) or vinculin (V9131, *Sigma-Aldrich*) and detected with Alexa 647- or Alexa 488-conjugated, isotype-specific, anti-IgG antibodies (*Invitrogen*). Actin was labeled with phalloidin-TRITC (*Sigma-Aldrich*) and nuclei were stained with DAPI (D1306, *Invitrogen*).

ALP was stained with fast blue RR salt in a 0.01 % (w/v) naphthol AS-MX solution (*Sigma Aldrich*) according to the manufacturer's instructions.

IV. Spectrometry

The release of BMP-2 from the patterns was evaluated by measuring the amount of BMP-2^{CF} present in the medium every 30 minutes for 4 hours at 37 °C with a UV–visible spectrometer

(Infinite M1000, *Tecan*). The excitation wavelength used was 492 ± 5 nm, and the emission wavelength was 517± 5 nm.

V. Confocal microscopy

Confocal images and fluorescence recovery after photobleaching (FRAP) experiments were conducted with a Zeiss LSM 700 confocal laser scanning microscope (*Zeiss*) equipped with a 63x oil immersion objective.

1. Imaging

Confocal images of isolated patterns were used to quantify the surface concentration of transferred BMP-2^{CF} thanks to a calibration curve obtained by UV-visible spectrometry using a microplate reader (Infinite M1000, *Tecan*) on PEM films homogeneously loaded with BMP-2 (Crouzier et al., 2009).

For cell behavior analysis, images of single cells spread on individual patterns were acquired using the appropriate channels depending on the immunofluorescent probes used.

2. Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were conducted to evaluate the possible diffusion of BMP-2^{CF} within $(PLL/HA)_{24}$ films. To this end, a 15 µm diameter circular region of interest (ROI) was bleached using the 488 nm laser diode, and the recovery after photobleaching was followed over time. The fluorescence of a similar but unbleached 15 µm diameter ROI on a separate pattern was also tracked over time as a control.

3. Force measurement

Polyacrylamide gels containing carboxylate-modified polystyrene fluorescent beads (F-8807, dark red 200 nm, *Invitrogen*) were used for force measurement in D1 murine MSCs (Vignaud et al., 2014). Concentrations of acrylamide and bisacylamide were chosen depending on the desired stiffness, and based on the experimental conditions described in the literature (Tse & Engler, 2010). After careful selection of fields containing single cells spread on patterns after 4 hours of culture, images of the fluorescent beads were acquired. Cell were then detached from the polyacrylamide substrate by adding 0.01 % sodium dodecyl sulfate to the medium, as previously described (Bergert et al., 2016). Once all the cells were detached, the same fields were imaged once again, and the displacement of the beads which occurred during cell detachment was used for force measurement and analysis, as explained below.

VI. Atomic force microscopy (AFM)

AFM images were obtained in PBS in peak force tapping mode using an AFM BioCatalyst instrument (*Bruker*). Pyramidal silicon nitride cantilevers (MSNL probes, *Bruker*) with a spring constant of 0.07 $N.m^{-1}$ were used. The analysis of the topography of 5 patterns per condition was performed using Nanoscope analysis (*Bruker*).

VII. Immunoblotting

Cells were lysed in Laemmli buffer (*Sigma-Aldrich*). Detection of proteins by Western blotting was done according to standard protocols. After electrotransfer and blocking (10 mM Tris at pH 7.9, 150 mM NaCl, 0.5 % Tween 20, and 3 % dry milk at room temperature

for 1 hour), the PVDF membrane was incubated with antibodies overnight at 4 °C. Immunological detection was achieved with horse radish peroxidase-conjugated secondary antibody. Peroxidase activity was visualized by enhanced chemiluminescence (ECL) (West pico signal; *Thermo Fisher Scientific*) using a ChemiDoc MP imaging system (*Bio-Rad Laboratories*). As a control, detection of actin or GADPH was also performed.

VIII. Image analysis

1. Pattern characterization

Images of individual patterns were analyzed using ImageJ 1.50g (*National Institutes of Health*). The area and overall intensity of the pattern were measured. All acquisition parameters being constant for all imaged patterns, these values could be compared and their analyses used to assess the efficiency of BMP-2 transfer onto the film depending on the printing conditions, as well as the faithfulness and reproducibility of the printing of the patterns.

2. p-SMAD1/5/8

Image averaging and p-SMAD1/5/8 quantification were performed using homemade Image J routines. For experiments requiring p-SMAD1/5/8 quantification, the following excitations wavelengths were used:

- for BMP-2^{CF} (pattern): 488 nm
- for phalloidin-TRITC (actin): 555 nm
- for DAPI (nucleus): 405 nm
- for Anti-RABBIT Alexa Fluor 647 (p-SMAD1/5/8): 639 nm

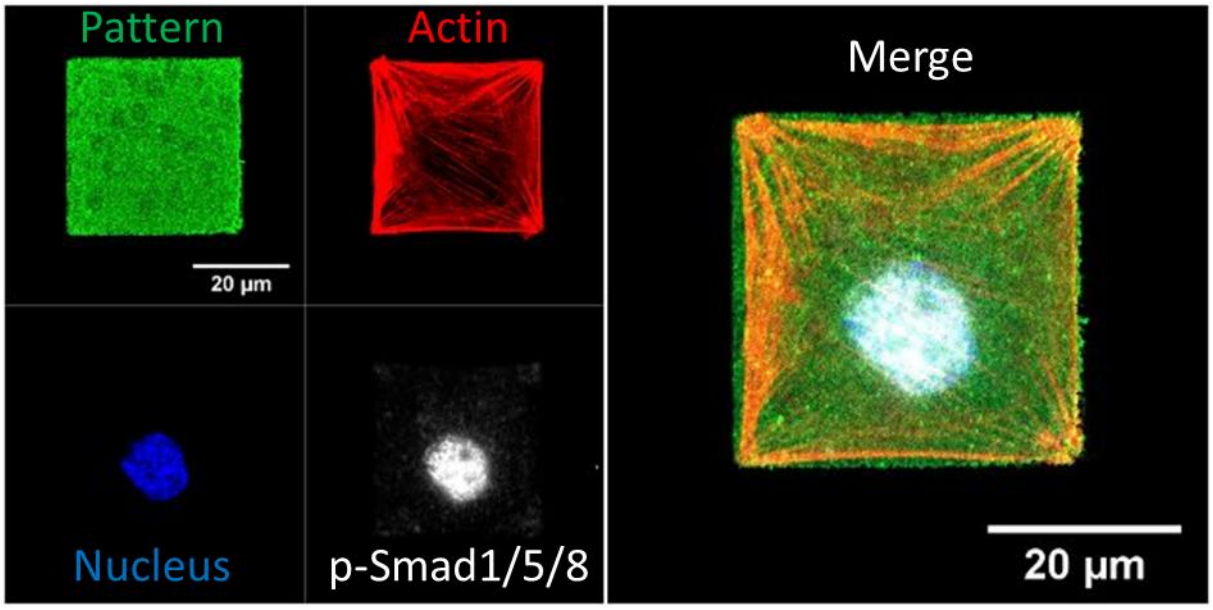


Figure 48: ***Representative images of a pattern, actin cytoskeleton, nucleus and p-SMAD1/5/8, obtained by immunofluorescent staining and confocal microscopy****.*

Nuclear and cytoplasmic p-SMAD1/5/8 fluorescence intensities were measured over the area of the nucleus and cytoplasm, respectively, obtained from binarized nucleus and actin images. To do so, a threshold was first applied to the actin image, and used to create a mask of the cell area.

A similar process was then applied to the nucleus image, to obtain a mask of the area of the nucleus.

These two masks were then overlaid on the image of p-SMAD1/5/8, and the fluorescent intensities in the cell and the nucleus were measured, as shown in Figure 49.

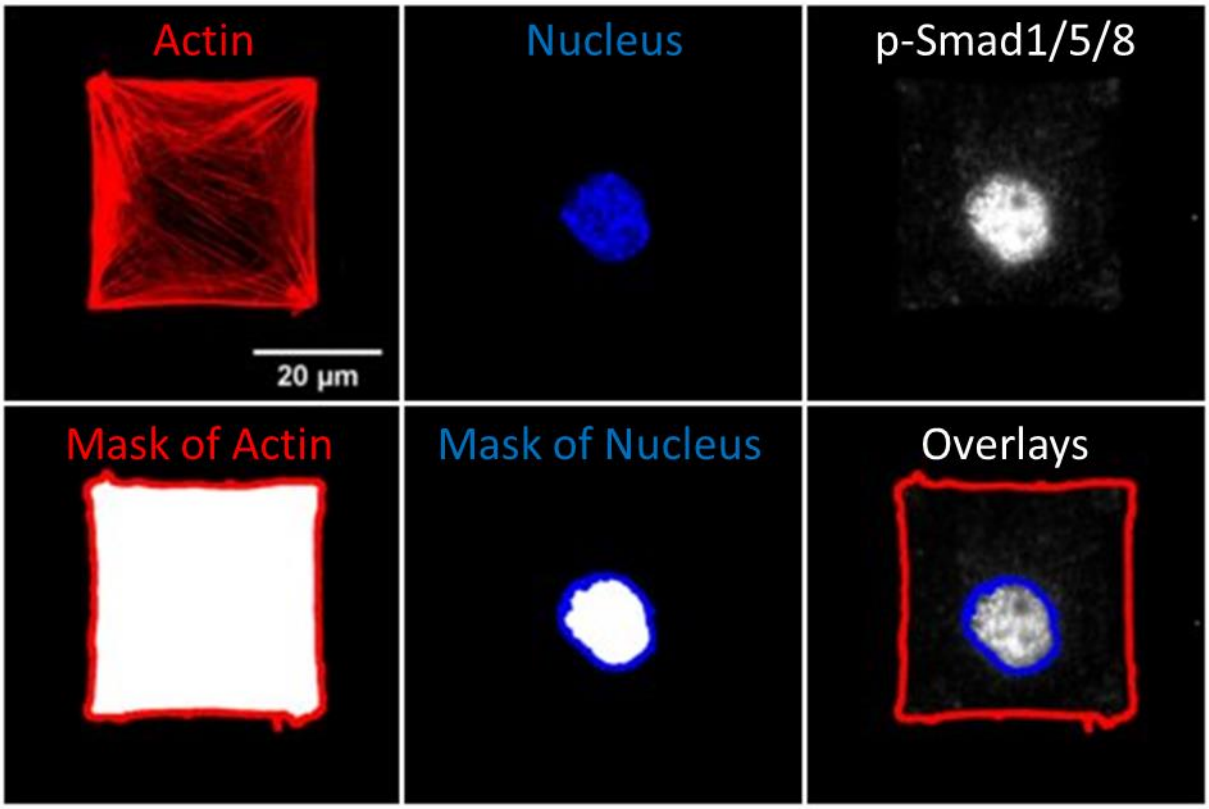


Figure 49: ***Representative images of actin and nucleus and their respective masks, used as overlays on the p-SMAD1/5/8 image to obtain the nuclear and cellular relative amount of p-SMAD1/5/8****.*

The areas and overall intensities measured for the nucleus and the entire cell were then used as a means of quantifying the relative nuclear versus cytoplasmic p-SMAD1/5/8 signal in the cell.

3. FRAP

The fluorescence intensity of the region of interest (ROI, in white) was normalized to that of an unbleached control region (in yellow), as shown in Figure 50. This allowed an adjustment of the recovery to outside conditions which could affect the measured signal (drift of the microscope objective in z, photobleaching due to successive acquisitions…).

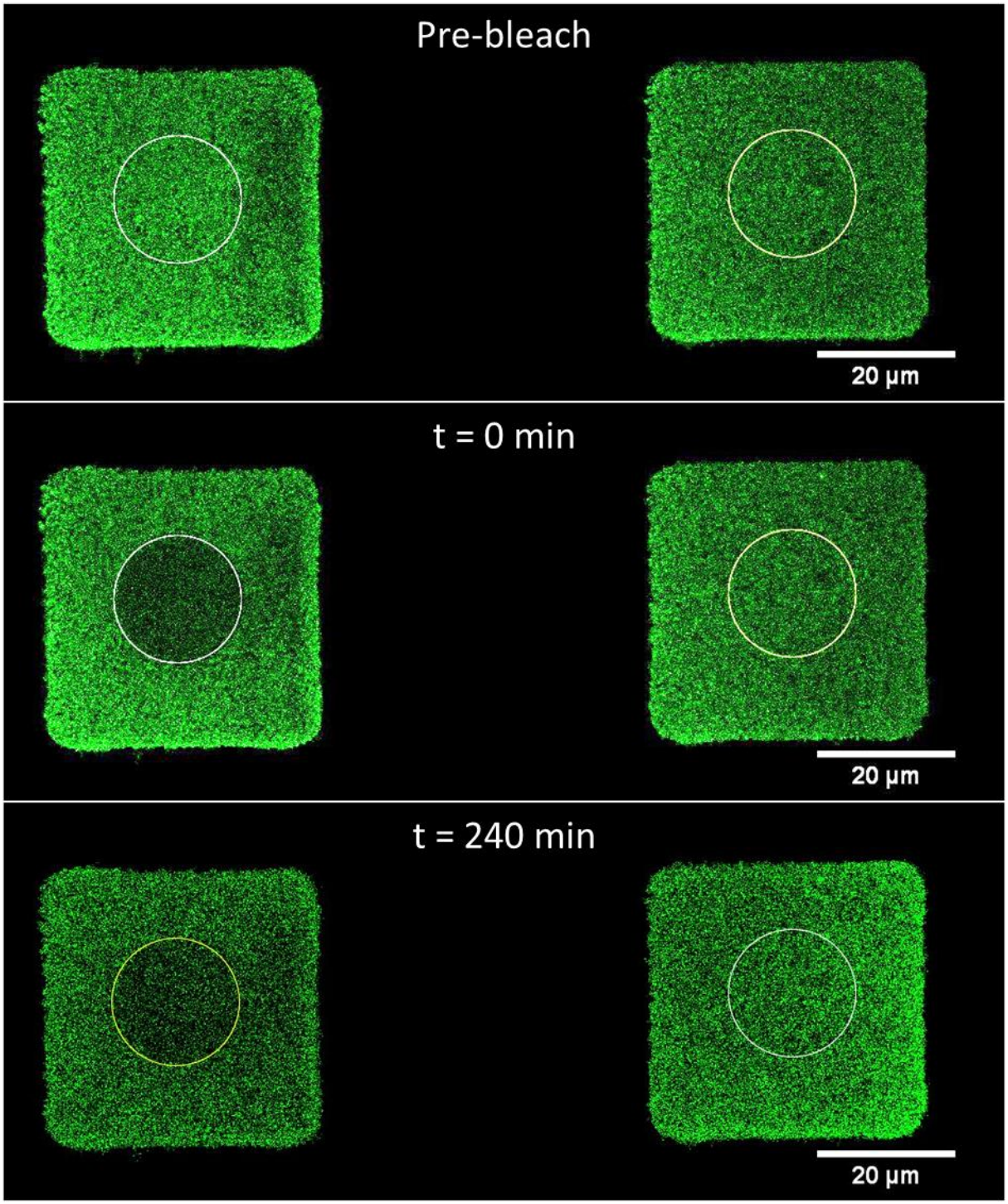


Figure 50: ***Representative images of patterns before bleaching, just after bleaching, and after 4 hours at 37 °C****.*

4. Directionality

Actin and fibronectin orientations were evaluated with the Directionality plugin (http://fiji.sc/wiki/index.php/Directionality) in Image J.

5. Actin organization in z

Actin organization in the z-axis was performed using homemade ImageJ routines. Briefly, a small region of interest was chosen in the center of the nucleus of each cell, and the intensity profile of the actin cytoskeleton and the nucleus in the z-axis were plotted. After normalization of the signal over the highest intensity, the data were centered over the slice of highest intensity of the nuclear signal, and averaged over the total number of cells. This allowed us to display the average intensity plot and localization of the actin cytoskeleton relative to the nucleus depending on the printing condition.

6. Force images analysis

The TFM images obtained on polyacrylamide gels were then used to measure bead displacement after cell detachment. Indeed, due to the polyacrylamide's elastic properties, the deformation of the substrate incurred by cell tension is reversed after cell detachment, and the beads return to their initial position. After correcting for experimental drift, this displacement can therefore be measured using the pre- and post-cell detachment images. This is done thanks to the generation of a displacement field using first particle image velocimetry, then individual bead tracking. The measured displacement can then be correlated with a total amount of force exerted by the cell on its substrate, as previously described (Butler, Tolić-Nørrelykke, Fabry, & Fredberg, 2002; Mandal, Wang, Vitiello, Orellana, & Balland, 2014; Sabass, Gardel, Waterman, & Schwarz, 2008; Tseng et al., 2011).

These experiments were carried out in the Chen Lab at Boston Univeristy, and force measurement calculations were carried out by custom-built Matlab protocols developed at

the Laboratoire Interdisciplinaire de Physique (LiPhy, Grenoble). All TFM results were obtained with the kind help of Tomas Andersen, Christopher Chen, Anant Chopra, Martial Balland and Irene Wang.

IX. Statistical analysis

For each histogram, the mean represents the average of three to five independent experiments ± standard deviation. Significances were assessed by one-way analysis of variance, using Tukey's honestly significant difference test to compare each pair of data.

RESULTS

I. Printing technique

We combined the techniques of layer-by-layer (LbL) deposition and microcontact printing in order to create micropatterns of BMP-2 on soft biopolymeric films.

By choosing a printing method which relies solely on the physical transfer of BMP-2 onto the substrate (*i.e.* microcontact printing), we avoided any chemical or conformational modification of the protein. This ensured that we were not affecting the biological activity of BMP-2 during the printing process.

By alternatively dipping glass coverslips in PLL and HA solutions, we obtained films comprised of 24 bilayers of Poly(L-Lysine) (PLL) and Hyaluronic Acid (HA), hereafter named (PLL/HA) films. These (PLL/HA) films were chosen for three very specific reasons.

- First, the level of crosslinking of these (PLL/HA) films can be controlled using carbodiimide chemistry to create covalent amide bounds between the amine groups of PLL and the carboxyl groups of HA. Films crosslinked with a carbodiimide solution at 30 $mg.mL^{-1}$ present a Young's modulus of ~200kPa (Boudou, Crouzier, Auzély-Velty, Glinel, & Picart, 2009; Schneider et al., 2008), which is of the same order of magnitude as the stiffness of precalcified bone *in vivo* (Discher et al., 2009; Halder et al., 2012).

- Secondly, it has previously been demonstrated in the team that that these films can retain BMP-2 and that C2C12 myoblasts cultured on (PLL/HA) films presenting matrix-bound BMP-2 are able to transdifferentiate into bone cells, as assessed by measuring alkaline phosphatase (ALP) activity *in vitro* (Crouzier et al., 2009). In addition, it has previously been shown that BMP-2 bioactivity is maintained within the films, even once dried, and that they are osteoinductive in a rat ectopic model *in vivo* (Raphael Guillot et al., 2013). They were recently shown to regenerate a critical size femoral defect in rat when deposited on Polylacticglycolic acid (PLGA) implants (Bouyer et al., 2016).

- Finally, the ability of cells to adhere and spread on these films strongly depends on the level of film crosslinking (Schneider et al., 2006). Indeed, cells cultured on slightly crosslinked (PLL/HA) films tend to stay round and poorly spread when no BMP-2 is loaded within the film or when BMP-2 is presented in solution. On the contrary, cells adhere and rapidly spread on the same films containing matrix-bound BMP-2 (Crouzier, Fourel, et al., 2011).

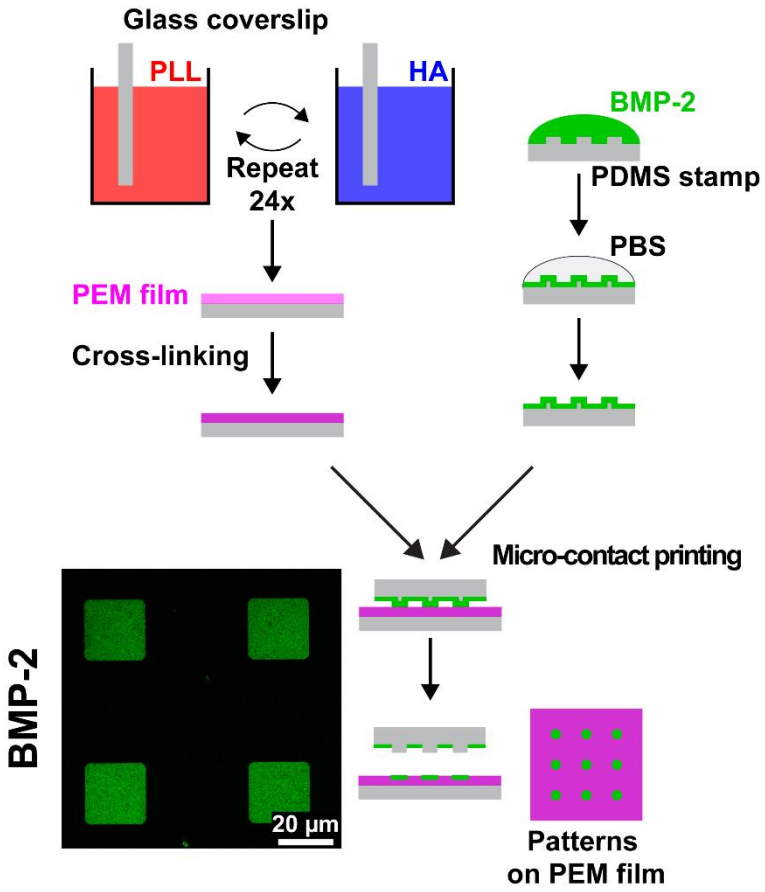


Figure 51 : ***Microcontact printing of BMP-2 on soft biopolymeric films****. Schematic of the LbL buildup and crosslinking of $(PLL/HA)_{24}$ films (upper left) combined with microcontact printing (upper right). The ink (in green) used for generating micropatterns on soft (PLL/HA) films contains 50 µg.mL^{-1} of BMP-2. Bottom left: representative image depicting the reproduction of the photomask's spatial features in BMP-2^{CF} micropatterns.*

As is shown in Figure 51, we were able to use microcontact printing to generate micropatterns of BMP-2 on these slightly crosslinked (PLL/HA) films by adsorbing this protein onto polydimethylsiloxane (PDMS) stamps, and placing these stamps in conformal contact with the film.

Visually, these patterns appeared to be well resolved, with well-defined and clear edges, and a homogeneous distribution. To confirm the validity of this visual assessment, we characterized precisely the yield of the protein transfer during the printing process, the stability of the micropatterns over time, and the spatial resolution we were able to obtain with this technique. From a biological standpoint, we investigated the bioactivity of BMP-2 micropatterns, in terms of adhesion, spreading, and early transdifferentiation, using C2C12 myoblasts.

II. Microcontact printing of BMP-2 alone on biopolymeric films

1. BMP-2 transfer yield

To quantify the amount of BMP-2 transferred during the printing process, we used confocal imaging of BMP-2 labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (BMP-2^{CF}) (Crouzier et al., 2009).

Using a BMP-2^{CF} loading concentration (*i.e.* concentration of BMP-2 in the ink deposited on the PDMS stamps) of 50 µg.mL^{-1}, we measured a BMP-2 surface concentration of 214 ± 85 ng.cm^{-2}, which is equivalent to a transfer yield of 4 %. This transfer yield was determined by calculating the ratio of BMP-2 transferred onto the surface over the quantity of BMP-2 in the drop of solution used during the PDMS loading step. We were able to determine the cause for this low transfer yield by collecting the drop after the loading step, and measuring the quantity of BMP-2 remaining in solution. Using fluorescence spectrometry, we were thus able to determine that the limiting transfer step is the low adsorption of BMP-2 on the PDMS stamp.

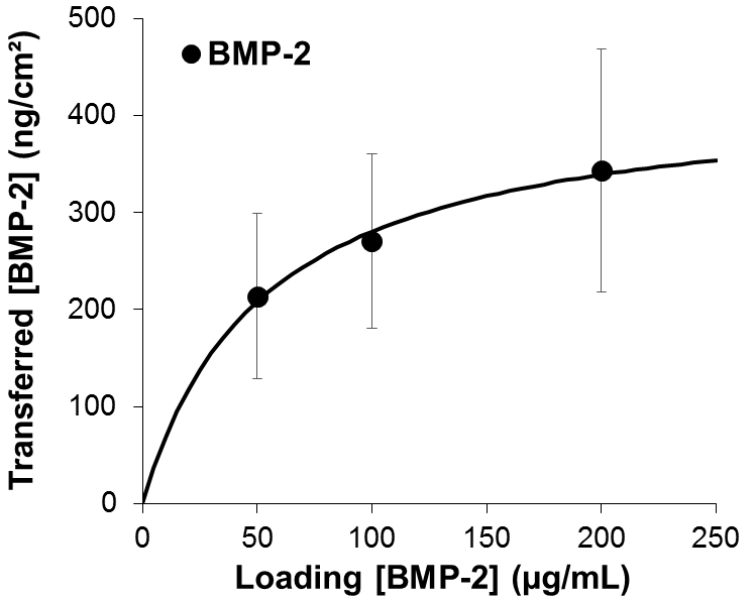


*Figure 52: **Transfer yield of BMP-2 micropatterns**. Quantification of the surface concentration of printed BMP-2^{CF}, as a function of the loading concentration of BMP-2^{CF} on the stamp, obtained by confocal microscopy. Error bars are the standard deviation over n=80 measurements. Fitting was carried out using a Michaelis-Menten function.*

It should be noted that the quantities of matrix-bound BMP-2 described above can be tuned by changing the loading concentration of BMP-2 (Figure 52). The surface density of BMP-2 alone can thus be slightly increased to 344 ± 125 ng.cm^{-2} by increasing the loading concentration of BMP-2 from 50 to 200 µg.mL^{-1}. Of note, for loading concentrations of 100 and 200 µg.mL^{-1}, the totalBMP-2/BMP-2^{CF} ratios were 2 and 4, respectively. This was done so as to be able to use the same acquisition parameters for all conditions without pixel saturation. Fluorescent intensities were then adjusted to total BMP-2 concentrations, and fitted using a Michaelis-Menten function.

We were therefore able to physically transfer BMP-2 onto (PLL/HA) films using microcontact printing, and to do so in a spatially controlled manner. We also showed that the amount of BMP-2 transferred onto the film depends on the BMP-2 loading concentration on the PDMS stamps. Spatial control being the cornerstone of micropatterning techniques, we then characterized the spatial resolution of the patterns.

2. Spatial resolution of BMP-2 micropatterns

Our goal in using micropatterning was to spatially control BMP-2 presentation, in a very precise manner, which is why we characterized the spatial resolution of BMP-2 micropatterns.

In images obtained using confocal microscopy, the BMP-2 patterns appeared well defined in the x and y directions, the edges were well resolved, and in the z direction the patterns appeared to sit on the surface of the (PLL/HA) film (Figure 53A). For this, and future experiments, we chose a loading concentration of BMP-2 of 50 µg.mL^{-1}, which corresponds to a surface concentration of BMP-2 of 214 ± 85 ng.cm^{-2} (Figure 52).

However, as the z-resolution of laser scanning confocal microscopy is limited to ~500 nm, we also imaged the micropatterns of BMP-2 using atomic force microscopy (AFM). As is shown in Figure 53C, we were thus able to confirm that the films were not damaged during microcontact printing, and we measured the thickness of the protein layer. BMP-2 patterns were 5 ± 1 nm thick (Figure 53D), meaning that only a mono- to bi-layer of BMP-2 was transferred onto the film, as the estimated size of a BMP-2 dimer is approximately 6.4 × 3.4 × 3.0 nm per dimer (Scheufler, Sebald, & Hülsmeyer, 1999).

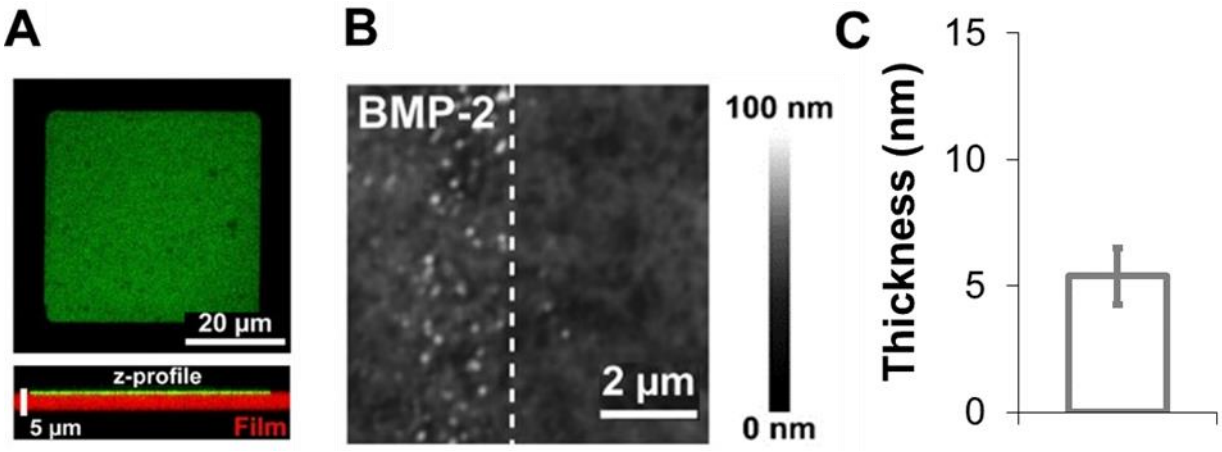


Figure 53 : ***Spatial characterization of BMP-2 micropatterns****. (A) Confocal images and cross-sections of the BMP-2^{CF} micropatterns. The patterns are in green and the film in red (for the cross-section only). (B) Printed amounts of BMP-2 in micropatterns. (C) High resolution AFM topography images and (D) thickness measurements of the protein layer on the border of micropatterns (located on the left of the image and suggested by a dashed line.*

We were thus able to successfully generate patterns of BMP-2 of several hundred square micrometers on (PLL/HA) films, a useful tool for single cell studies. Furthermore, we wished to see what resolution we could obtain using microcontact printing on PEM films, and if this technique could also be used for the study of subcellular processes.

As is shown in Figure 54, the use of a photoresist designed for high aspect ratios allowed a faithful reproduction of the photomask's spatial features and ensured a good subcellular spatial resolution, close to 1.5 µm², which is comparable to resolutions obtained for microcontact printing on stiff substrates (Théry & Piel, 2009).

We were able to confirm this by plotting the fluorescence profile of BMP-2^{CF} of a line of 10 dots, part of a 10-by-10 dot array printed over a total square area of 1500 µm².

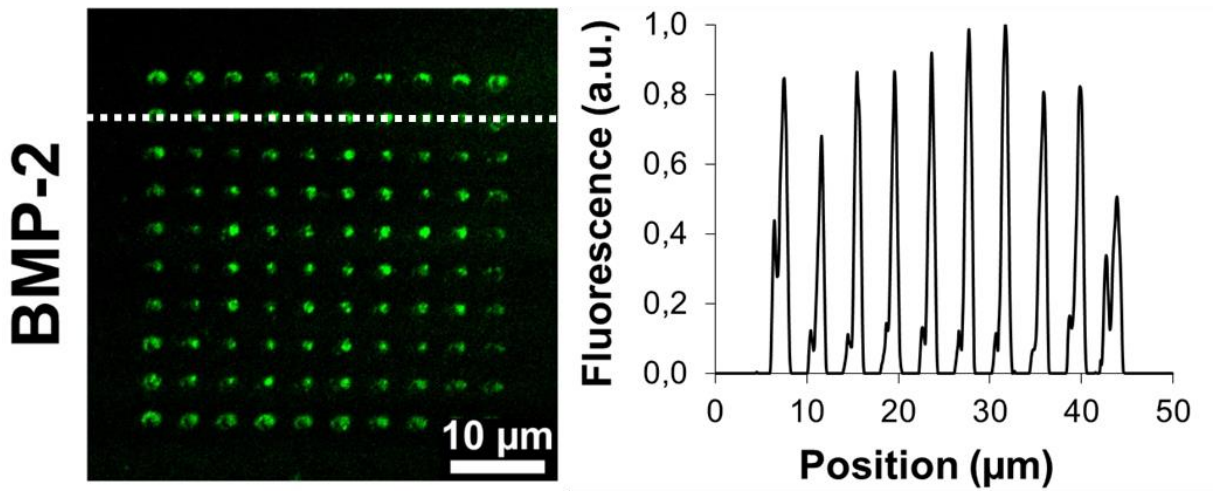


Figure 54 : ***Subcellular sized micropatterns****. Representative images of high-resolution BMP-2 micropatterns with a dot average area of 1.49 ± 0.47 µm² and fluorescence profile along one line of dots of the BMP-2 image, highlighting the spatial resolution of the micropatterns.*

It should be noted that all measurements were performed after extensive rinsing steps of the patterned films, our goal being that only immobilized BMP-2 should remain on the (PLL/HA) films. Since our interest in printing BMP-2 on (PLL/HA) films is to present this protein in a matrix-bound manner, an important step was to check that the BMP-2 we had

transferred onto the film was indeed immobilized on the pattern, and not being released in solution or diffusing within the film.

3. BMP-2 immobilization

To verify the stability of the BMP-2 micropatterns, and that BMP-2 was truly immobilized on the film, we measured the diffusion of BMP-2 using fluorescence recovery after photobleaching (FRAP) on patterns printed with BMP-2 labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (BMP-2^{CF}). We also made sure that BMP-2^{CF} was not released from the micropatterns by quantifying its concentration in solution over the course of 4 hours, by fluorescence spectroscopy.

For both these experiments, the patterns were left at 37 °C between measurements, in order to guarantee conditions as close as possible to those during cell culture. They were also left in the dark to avoid any undesired photobleaching which could affect measurements.

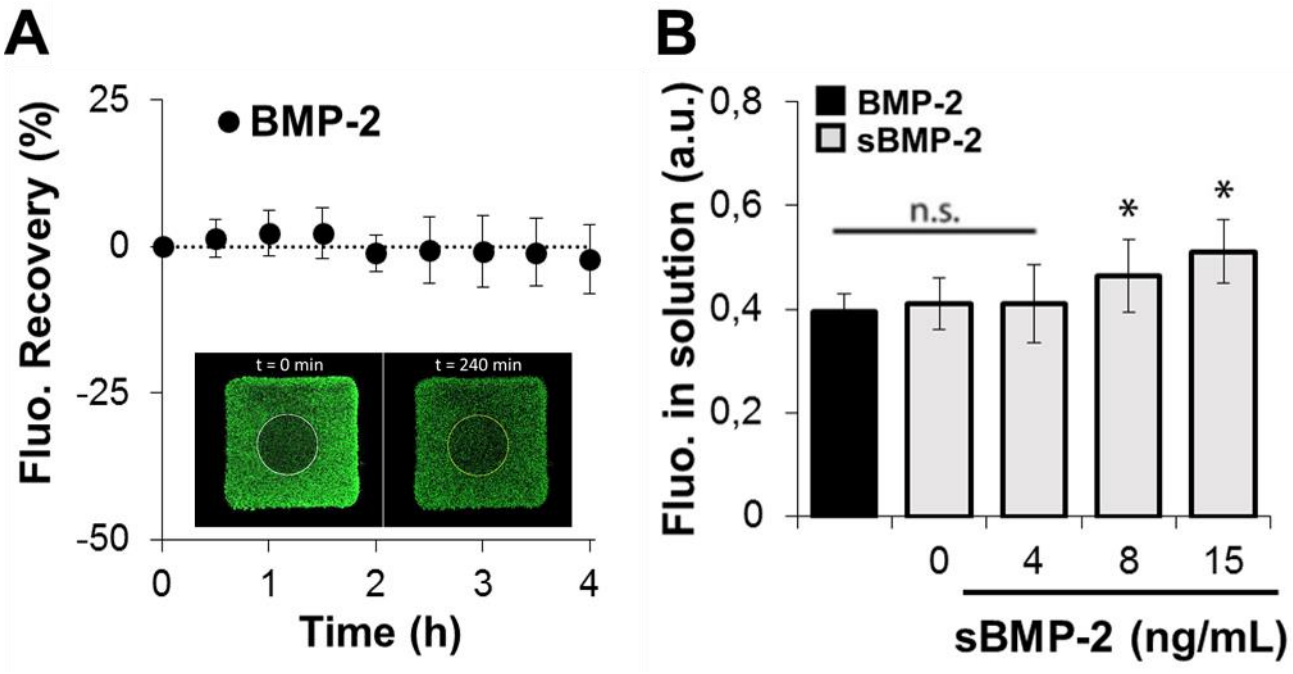


*Figure 55 : **BMP-2 is immobilized when microprinted**. (A) Fluorescence recovery after photobleaching of BMP-2^{CF} in BMP-2 micropatterns for 4 hours. (B) Fluorescence of BMP-2^{CF} released in solution from BMP-2 micropatterns after 4 hours, compared with soluble BMP-2^{CF} (sBMP-2^{CF}) at concentrations ranging from 0 to 15 ng.mL-1 (light gray). * $p < 0.05$ versus negative control (no sBMP-2^{CF}).*

As can be seen in Figure 55, no recovery of fluorescence was observed 4 hours after photobleaching, indicating the absence of diffusion of BMP-2 within the pattern.

Similarly, we could not measure any significant fluorescence of BMP-2^{CF} in solution after 4 hours at 37 °C, meaning that, if present, the BMP-2 concentration in solution was less than 8 ng.mL^{-1}, which is known to be too low to trigger osteogenic transdifferentiation (Crouzier et al., 2009; Katagiri et al., 1994; Schwab et al., 2015).

It can therefore be assumed that any effect of the BMP-2 patterns on cell behavior is due exclusively to the matrix-bound presentation of this protein, and not to the presentation of BMP-2 in solution due to a release of this protein in the surrounding cell culture medium.

Once we had confirmed that the protein transfer was occurring during the printing process, that the microcontact printing technique was allowing us to efficiently control the spatial presentation of BMP-2 in an immobilized manner, and that the film remained intact during the entire process, our goal was to check if BMP-2 printed in this manner remained bioactive, and to investigate how cells responded to these patterns, in particular in terms of adhesion, spreading and differentiation.

4. Bioactivity of BMP-2 in micropatterns

Following the physical characterization of BMP-2 micropatterns, we therefore looked more closely at the biological relevance of this presentation mode. The first step was to make sure that BMP-2 immobilized in micropatterns on (PLL/HA) films maintained its bioactivity, and in particular its osteoinductive properties, during the printing process.

The effect of BMP-2 on C2C12 murine myoblasts, and in particular its ability to induce ALP activity and the activation of the SMAD signaling pathway, is very well documented and characterized (Fourel et al., 2016; Heldin, Miyazono, & ten Dijke, 1997; Katagiri et al., 1994; F. Liu et al., 1996; J Massagué, 1998; Sieber et al., 2009). From a broader perspective, muscle

stem cells play a crucial role during bone regeneration in vivo, as demonstrated in animal models (Abou-Khalil et al., 2015) as well as in reconstructive surgery through the use of muscle flaps (Yazar, Lin, Lin, Ulusal, & Wei, 2006). It is for these reasons that we chose C2C12 myoblast transdifferentiation to the osteoblast lineage as a model to study the bioactivity of BMP-2 micropatterns on (PLL/HA) films.

As shown in Figure 56, our BMP-2 micropatterns are very stable over time, and can thus be used to study long term transdifferentiation events, such as the expression of the osteogenic marker alkaline phosphatase (ALP) (Katagiri et al., 1994) after 4 days of culture (Figure 56).

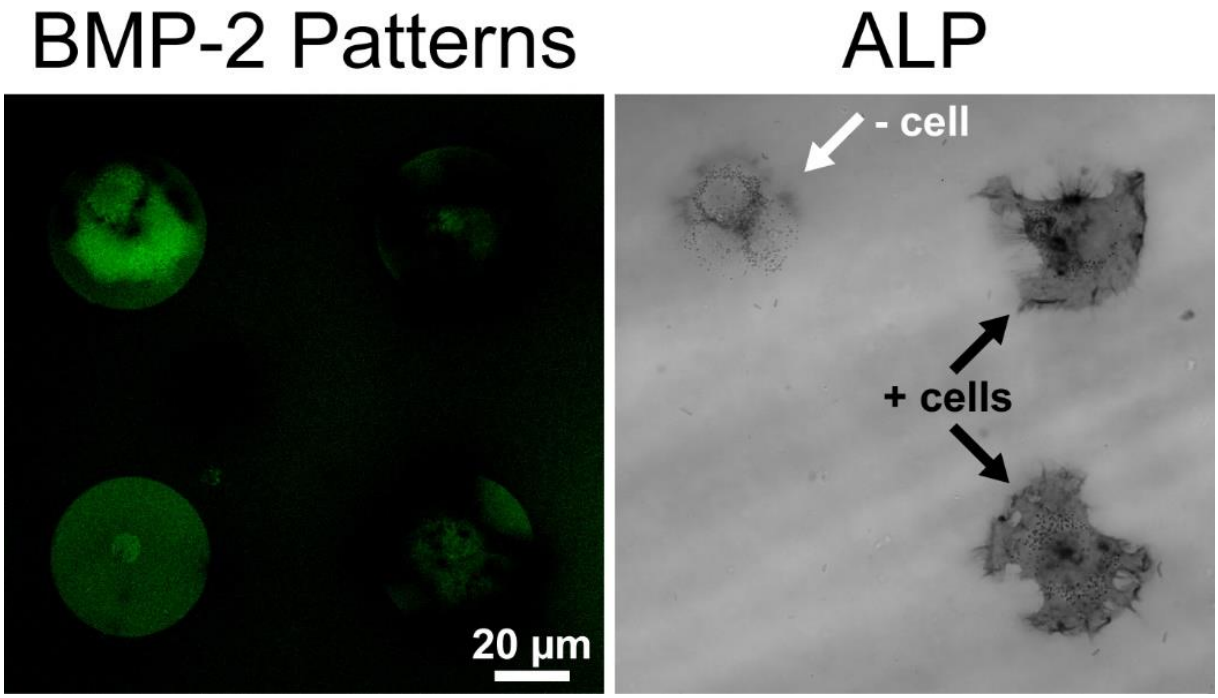


Figure 56: ***Long term culture and transdifferentiation of C2C12 myoblasts on BMP-2 micropatterns****. ALP staining in C2C12 myoblasts cultured on BMP-2 (in green) micropatterns for 4 days. Two cells are ALP positive (black arrows) and one is negative (white arrow). Note that the fluorescence of occupied micropatterns is absorbed by the ALP-staining.*

Nevertheless, our present study aimed at studying the behavior of single cells confined by the micropatterns, whereas when observing cells which had proliferated over 4 days of culture we could find very few micropatterns containing only a single cell.

We therefore focused on early transdifferentiation signaling by analyzing the phosphorylation and translocation of SMAD1/5/8 after 4 hours of culture. SMAD1/5/8 is a

protein known to play a key role in the transduction pathway from BMP-2 receptors to the nucleus (Heldin et al., 1997; F. Liu et al., 1996; J Massagué, 1998; Sieber et al., 2009). SMAD-mediated gene transcription requires SMAD1/5/8 phosphorylation, complex formation with SMAD4, and translocation of the complex into the nucleus where it acts as a transcription factor (F. Liu et al., 1996). We thus examined by immunofluorescence whether SMAD1/5/8 phosphorylation and translocation to the nucleus were impacted by the matrix-bound presentation of BMP-2.

The presentation of BMP-2 by a (PLL/HA) film triggered the phosphorylation of SMAD1/5/8 and its translocation into the nucleus, as can be seen in Figure 57.

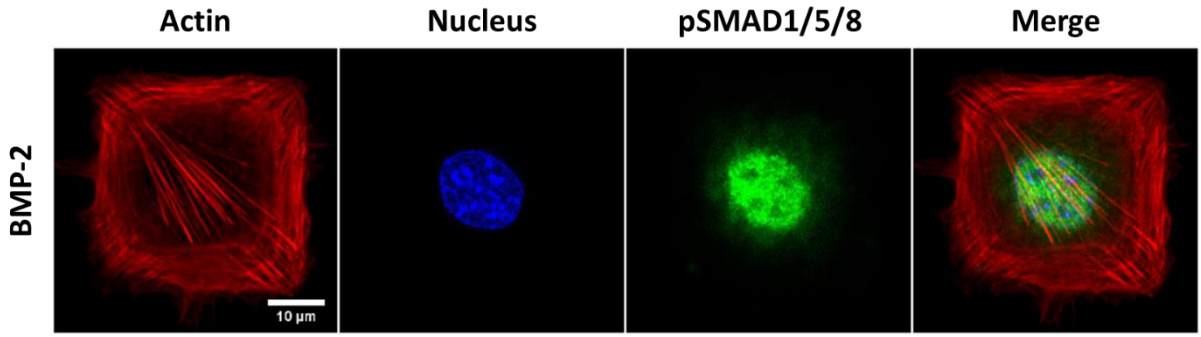


Figure 57: ***Micropatterned BMP-2 induces SMAD signaling****. Immunofluorescence images of C2C12 myoblasts spread on small (500 µm²) and large (1500 µm²) micropatterns of BMP-2 after 4 hours of culture, showing nuclear enrichment of p-SMAD1/5/8. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green.*

We thus confirmed, using C2C12 myoblasts, that BMP-2 presented by a micropattern on soft biopolymeric films was able to induce SMAD signaling. As we also wanted to control cell size using these patterns, we then looked more closely at cell adhesion and spreading on BMP-2 micropatterns.

5. C2C12 myoblast adhesion and spreading on BMP-2 micropatterns

As mentioned above, our team had previously demonstrated that matrix-bound BMP-2, and not BMP-2 in solution, could rescue C2C12 myoblast adhesion on otherwise non-adhesive slightly crosslinked (PLL/HA) films (Crouzier, Fourel, et al., 2011; Fourel et al., 2016).

By microcontact printing patterns of BMP-2 on these films, we were able to generate cell-adhesive micropatterns on an otherwise non-cell-adhesive surface.

Indeed, when we seeded C2C12 myoblasts on our micropatterned films, we observed a very selective adhesion of the cells only on the patterns of BMP-2, whereas almost no cells were observed outside of the patterns (Figure 58). This property allowed us to seed cells immediately after the printing process, without requiring an additional step of surface passivation to render the substrate surface non cell adhesive.

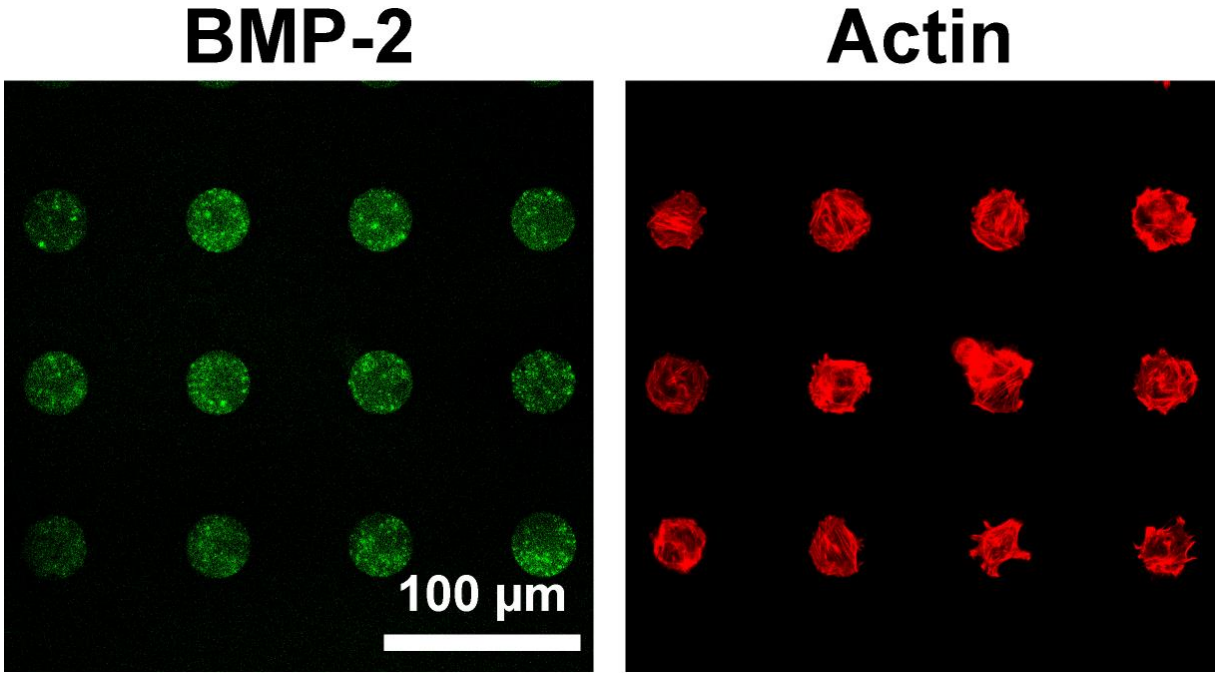


Figure 58 : ***Selective adhesion of C2C12 myoblasts on the micropatterns****. Representative images of BMP-2 patterns (in green) and of C2C12 myoblasts (actin staining in red) highlighting the very selective adhesion of the cells on the patterns, whereas almost no cells adhere outside of the patterns.*

We studied two different sizes of patterns, namely 500 µm² and 1500 µm² micropatterns. These values were chosen based on the areas described in the literature for C2C12 myoblasts (Crouzier, Fourel, et al., 2011). On BMP-2 micropatterns, we observed that C2C12 cells spread relatively well over 500 µm² patterns, but were not always able to cover the entire surface of 1500 µm² patterns. Our goal in this was to investigate the effect of cell spreading on BMP-2-induced signaling.

Figure 59 shows us a small sample of the manners in which cells responded to 1500 µm² BMP-2 patterns. On the graph, we can see that pattern size is highly normalized (504 ± 18 µm² and 1495 ± 61 µm² for 500 and 1500 µm² patterns, respectively). Cell area, on the other hand, is much more variable (337 ± 85 µm² and 942 ± 230 µm² for 500 and 1500 µm² patterns, respectively), although it does appear to be "capped" by pattern area (no cells were able to spread beyond the confines of the pattern). We were therefore able to restrict cell spreading to a maximum area (that of the pattern), but unfortunately not precisely control cell area.

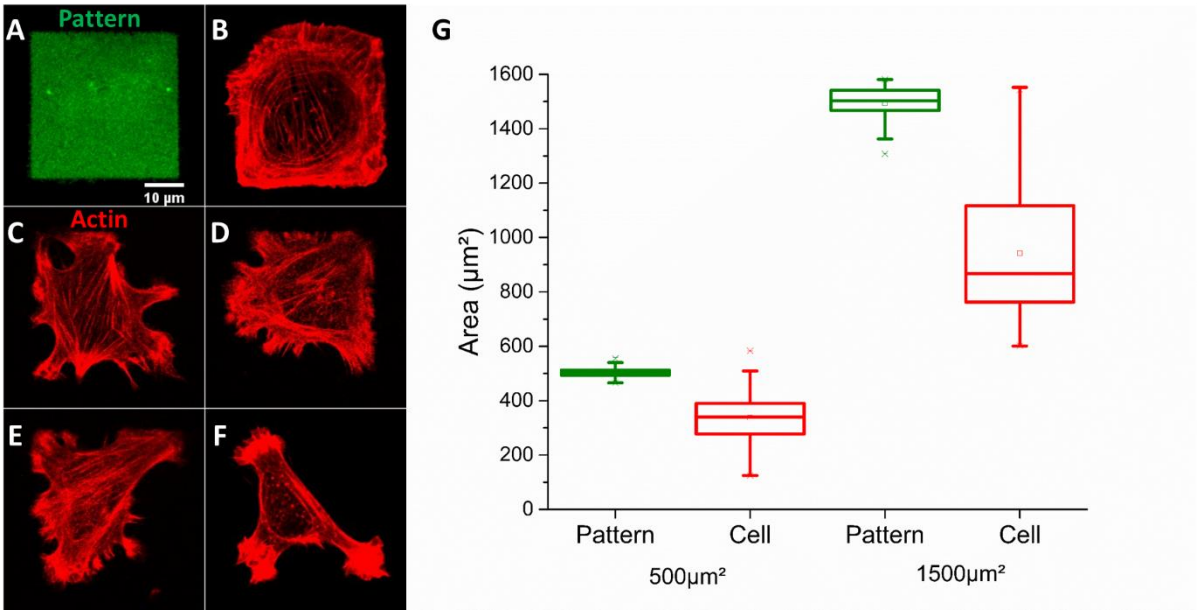


Figure 59: ***Efficiency of using patterns to control cell area*** *(A) Representative image of a pattern of BMP-2 on a (PLL/HA) film (B-F) Representative images of cells spread on BMP-2 micropatterns. (G) Quantification of pattern and cell areas for 500 µm² (n=112 and n=77, respectively) and 1500 µm² (n=95 and n=134, respectively) square patterns.*

Regarding the reasons for this inability of the BMP-2 patterns to control cell spreading, we posit that while matrix-bound BMP-2 can rescue cell adhesion on (PLL/HA) films, this BMP-2-mediated adhesion is not very effective for sustaining large spreading, especially considering the low amount of transferred BMP-2 when printed alone.

Despite this lack of normalization of cell areas on BMP-2 micropatterns, there were still significant differences in overall cell spreading between the small (500 µm²) and large (1500 µm²) patterns, and this ability to generate single cell-size micropatterns allowed us to

control cell spreading, and therefore investigate potential differences between unspread and spread cells.

While these results were promising, the significant issue regarding cell spreading – mentioned above – still needed to be resolved. Indeed, our goal was to use micropatterns to truly normalize cell spreading, and while there were non-negligible differences in cell areas on the small versus large micropatterns, the variations in cell size between these two conditions were still quite significant.

To correct this issue, we hypothesized that we could use to our advantage the high affinity of FN for several growth factors, including BMP-2, which had previously been described (Fourel et al., 2016; Mikael M Martino et al., 2014; Mikaël M Martino & Hubbell, 2010). The results of an enzyme-linked immunosorbent assay demonstrating this affinity are shown in Figure 16.

Keeping in mind that FN adsorbs well on PDMS (Toworfe, Composto, Adams, Shapiro, & Ducheyne, 2004), our hypothesis was that by combining FN and BMP-2, we would be able to both increase the transfer yield of BMP-2 onto the soft biopolymeric films, and allow cells to adhere and spread more efficiently on the micropatterns.

III. Microcontact printing of BMP-2 and fibronectin on biopolymeric films

1. Pattern quality and BMP-2 transfer yield

a. Pattern quality

The printing technique used for BMP-2 mixed with fibronectin (FN) was the same as the one described in section 1 for patterns of BMP-2 alone. With the goal of presenting BMP-2 in a spatially controlled manner while also controlling cell spreading, we once again combined the techniques of LbL deposition and microcontact printing, only this time to create micropatterns of unmodified BMP-2 mixed with FN on (PLL/HA) films.

To generate these micropatterns of BMP-2, we adsorbed a protein solution containing a combination of BMP-2 and FN at equal concentrations (*i.e.* 50 µg.mL^{-1}) on a PDMS stamp before transferring this adsorbed layer of proteins onto a slightly crosslinked (PLL/HA) film.

As is shown in Figure 60, we thus obtained micropatterns of BMP-2-containing FN (hereafter called FN/BMP-2) on biopolymeric (PLL/HA) films, thanks to the affinity between BMP-2 and FN (Martino et al., Science 2014; Martino et al., FASEB 2010; Fourel et al, JCB 2016), and the adsorption of FN on PDMS (Toworfe et al., 2004).

We also printed micropatterns of FN alone on (PLL/HA) films as negative (without soluble BMP-2) and positive (with soluble BMP-2, hereafter called sBMP-2) control conditions.

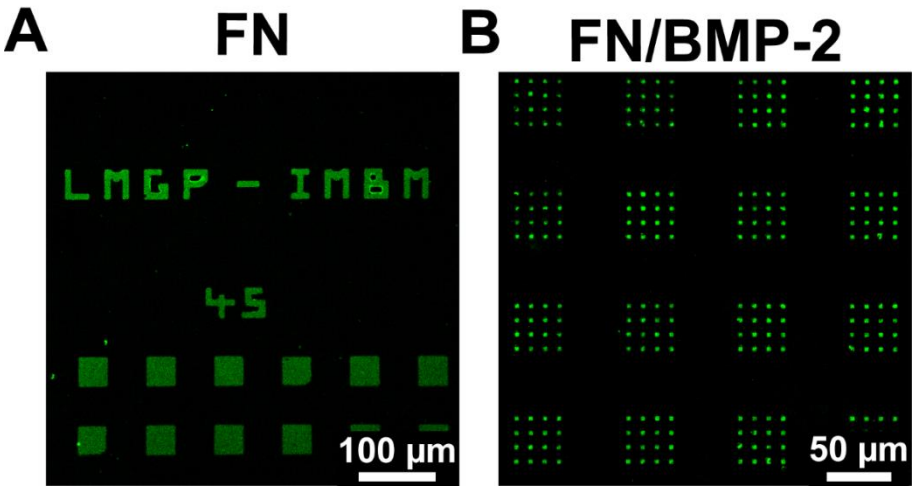


Figure 60: ***Microcontact printing of FN and FN/BMP-2 on soft biopolymeric films****. Representative images depicting the reproduction of the photomask's spatial features in FN (A) and FN-BMP-2 (B) micropatterns.*

b. BMP-2 transfer yield

Using the same techniques of fluorescence spectrometry and confocal imaging described above, we once again quantified the amount of patterned BMP-2 for different loading concentrations on the stamp (Figure 61).

We measured a BMP-2 surface concentration ranging from 700 ± 200 ng.cm^{-2} to 1300 ± 300 ng.cm^{-2} when the loading BMP-2 concentration is increased from 50 to 200 µg.mL^{-1}, with a constant loading FN concentration of 50 µg.mL^{-1}. As is shown in Figure 61, this represents a

significant increase in BMP-2 loading compared to patterns of BMP-2 alone (without FN). For a BMP-2 loading concentration of 50 µg.mL^{-1} for example, this represents a transfer yield of 14% (versus 4% for BMP-2 alone). Mixing BMP-2 and FN thus allowed us to obtain a three-fold increase in BMP-2 affinity, due to the high affinity of BMP-2 for FN (Mikaël M Martino & Hubbell, 2010), and the good adsorption of FN on PDMS (Toworfe et al., 2004).

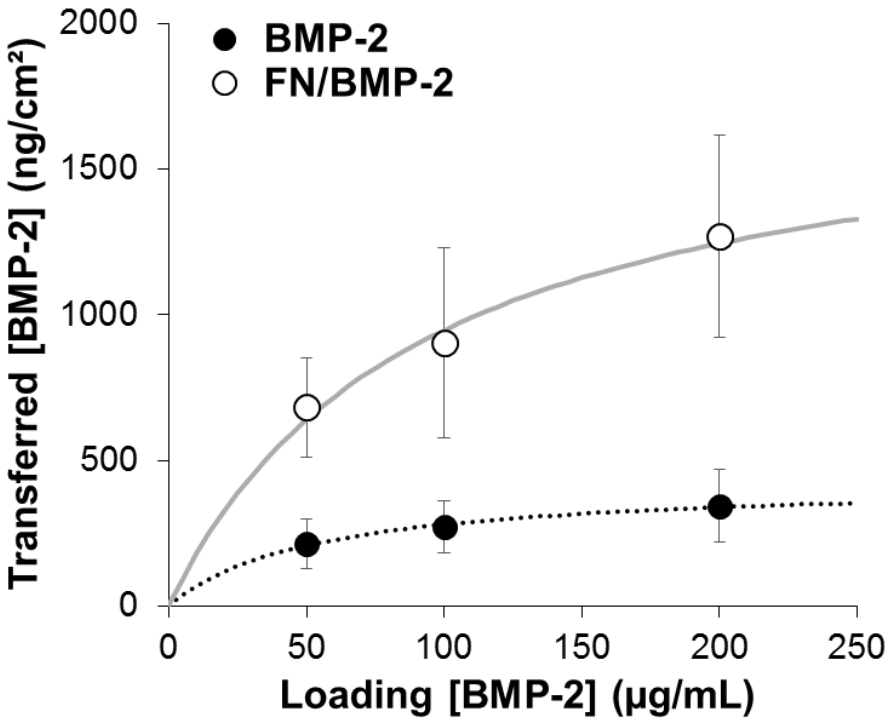


Figure 61: ***Transfer yield of FN/BMP-2 micropatterns in comparison to that of BMP-2 alone****. Quantification of the surface concentration of printed BMP-2 as a function of the loading concentration of BMP-2 on the stamp. The dashed line corresponds to the transfer yield of BMP-2 when printed alone, as a point of reference.*

With the goal of optimizing the printing process, we also investigated the effect of film dryness on the amount of BMP-2 transferred onto the film. As can be seen in Figure 62, drying the film with a flow of sterile nitrogen (Dry) or printing directly on the film without drying it (Humid) did not affect the amount of BMP-2 transferred onto the film, regardless of BMP-2 concentration.

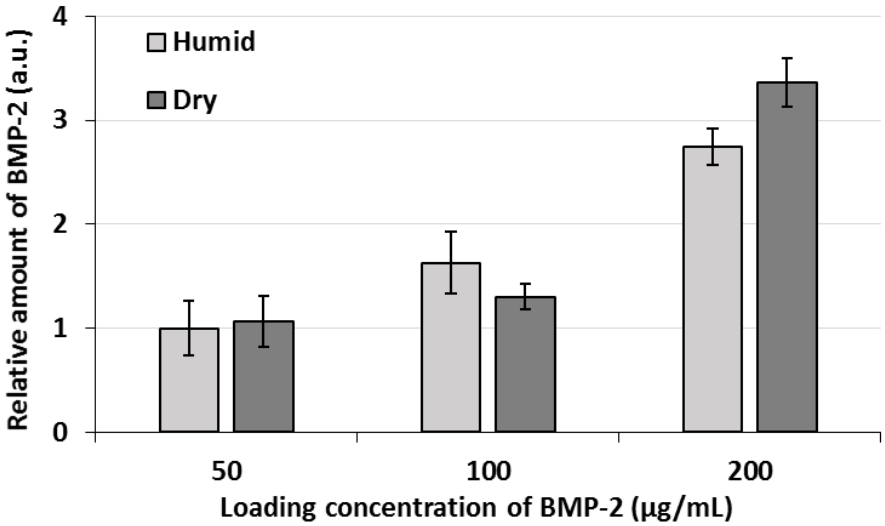


Figure 62: ***Comparison of BMP-2 transfer depending on film humidity****. Relative amount of BMP-2^{CF} in patterns of FN/BMP-2, as a function of BMP-2 loading concentration (50, 100 or 200 µg.mL-1), and of film dryness.*

It should however be noted that undesired printing effects due to capillarity were increased on humid films, thus resulting in printed regions outside of the desired patterned areas. For all future experiments, we therefore dried all films with a flow of sterile nitrogen prior to printing.

2. BMP-2 immobilization

With the experimental conditions – and in particular the ink composition – having changed, it was important to check that BMP-2 was still immobilized within the pattern when printed with FN, and not diffusing nor being released in solution.

As with BMP-2 alone, all experiments were performed several hours after extensive rinsing of the patterned films, so that only immobilized BMP-2 and FN remained. BMP-2 did not diffuse within the FN/BMP-2 micropatterns, as can be seen in Figure 63A by the absence of BMP-2^{CF} fluorescence recovery after photobleaching over a 4 hour period at 37 °C.

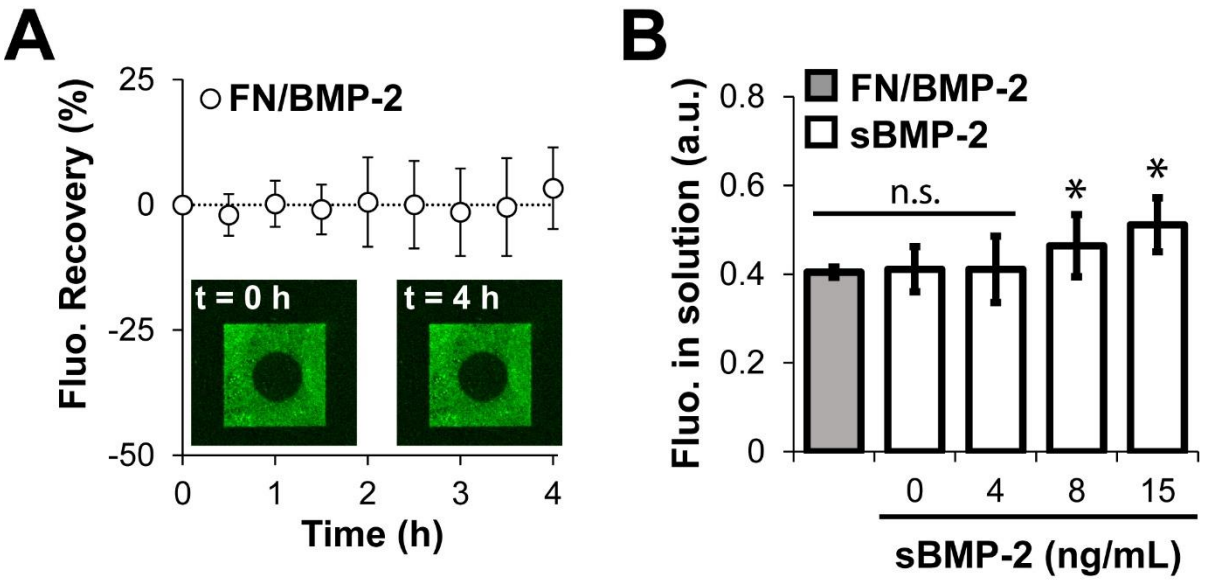


*Figure 63: **BMP-2 is immobilized when microprinted within FN**. (A) Fluorescence recovery after photobleaching of BMP-2^{CF} in a FN/BMP-2 micropatterns over 4h. (B) Fluorescence of BMP-2^{CF} released in solution from FN/BMP-2 (dark gray) micropatterns after 4h at 37 °C, compared with sBMP-2^{CF} at concentrations ranging from 0 to 15 ng mL-1 (light gray). * p < 0.05 versus negative control (no sBMP-2).*

Similarly, we did not measure any significant fluorescence of BMP-2^{CF} in solution after 4 h at 37 °C (Figure 63B), indicating that any quantity of BMP-2 released from the micropatterns was too low to trigger osteogenic transdifferentiation.

In the context of the study of BMP-2, we still needed to verify that printing this osteoinductive protein with FN did not affect its bioactivity. As we had done with the patterns of BMP-2 alone, we used the ability of C2C12 myoblasts to transdifferentiate toward an osteogenic lineage, and more specifically their ALP activity and the early activation of SMAD signaling (Fourel et al., 2016; Heldin et al., 1997; Liu et al., 1996; Massagué, 1998; Sieber et al., 2009).

3. Spatial resolution of FN and FN/BMP-2 micropatterns

Using these new printing conditions, we once again checked that the films were not damaged during the microcontact printing of FN or FN/BMP-2 (Figure 64). A confocal view of cross-sections of micropatterned films showed that the films were about 4 µm thick, with a very thin layer of proteins on top of it, and unmodified by the printing step (Figure 64A).

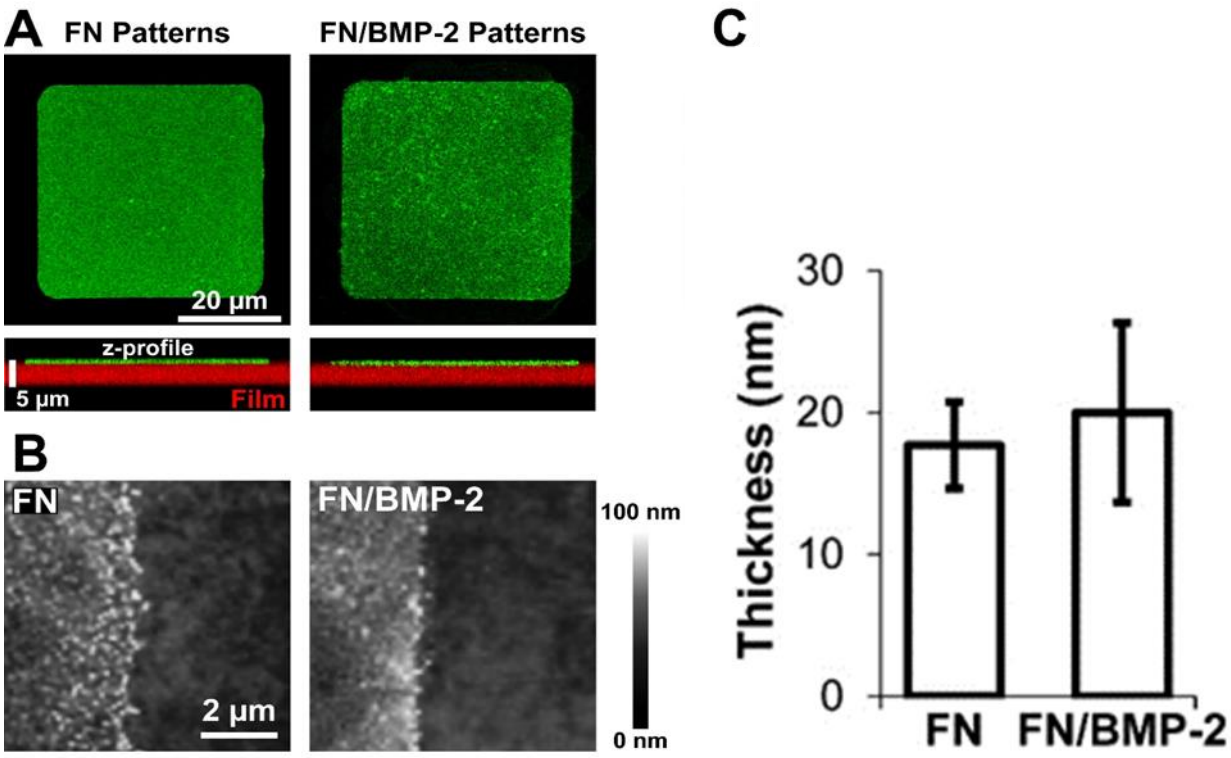


Figure 64: ***Characterization of FN/BMP-2 and FN micropatterns****. (A) Confocal images and cross-sections of micropatterns of FN (containing 10% of fibrinogen^{A488}) and FN/BMP-2^{CF}. The patterns are in green and the film in red (for the cross-section only). (B) High resolution AFM topography images and (C) thickness measurements of the protein layer on the border of micropatterns (located on the left of each image).*

We also imaged micropatterns of FN/BMP-2 and FN by atomic force microscopy (AFM) (Figure 64C). We were thus able to confirm that the films remained intact during microcontact printing, and to measure the thickness of the protein layer. FN/BMP-2 and FN patterns were both close to 20 nm thick, suggesting that BMP-2 dimers, with an estimated size of approximately 6.4 × 3.4 × 3.0 nm per dimer (Scheufler et al., 1999), were buried within a mesh of FN strands, which were shown to be approximately 120 x 2 x 2 nm (Erikson, Carrell, & McDonagh, 1981).

Fibronectin being a much bigger protein than BMP-2, we wanted to make sure that printing FN/BMP-2 did not affect the resolution we had previously been able to obtain when printing BMP-2 alone.

To this end, we printed a 10-by-10 array of small dots, spread evenly over a 1500 µm² square area. Once again, we were able to obtain an extremely faithful reproduction of the photomask's spatial features and subcellular spatial resolution.

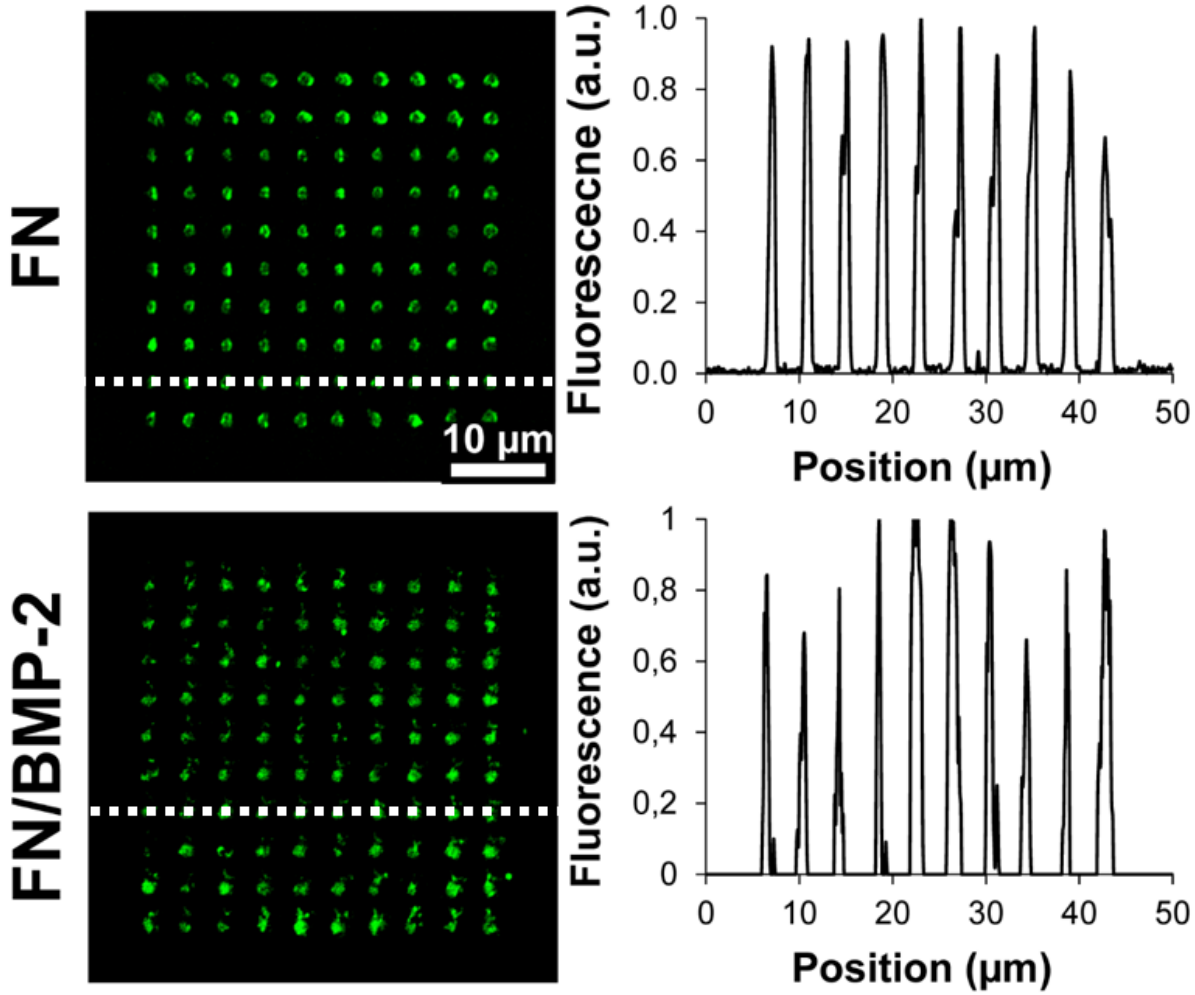


Figure 65: ***Subcellular micropatterns****. Representative images of high-resolution FN and FN/BMP-2 micropatterns with a dot average area of 2,16 ± 0,42 µm² and 1.74 ± 0.71 µm², respectively, and fluorescence profiles along one line of dots of the fluorescence image, highlighting the spatial resolution of the micropatterns.*

This subcellular resolution can be viewed by plotting the fluorescent intensity over a line of this 10-by-10 dot array. As is shown in Figure 65, and despite slight differences in dot area and fluorescent intensity, the printed areas are well resolved.

4. Bioactivity of BMP-2 in FN-bound BMP-2 micropatterns

As we had done for patterns of BMP-2 alone, we checked that BMP-2, when printed in combination with FN, maintained its ability to transdifferentiate C2C12 myoblasts toward an osteogenic lineage.

The micropatterns of FN/BMP-2 remained stable and bioactive for at least 4 days, as evidenced by the expression of the osteogenic marker alkaline phosphatase (ALP) (Katagiri et al., 1994), shown in Figure 66.

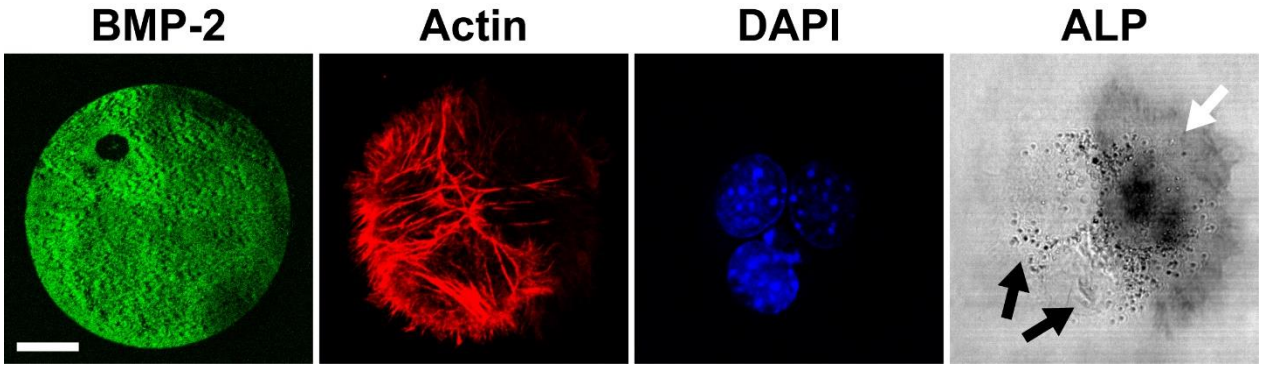


Figure 66: ***Long term culture and transdifferentiation of C2C12 myoblasts on FN/BMP-2 micropatterns****. Representative images of C2C12 myoblasts cultured on FN/BMP-2 (in green) micropatterns for 4 days. Actin is in red, nuclei in blue and the histochemical staining of ALP is dark in the positive cell (white arrow) and non-apparent in the two negative cells (black arrows). Note that the fluorescence of the positive cell is absorbed by the ALP staining. Scale bar is 10 µm.*

As had been the case for patterns of BMP-2 alone, this shows the ability of FN/BMP-2 patterns to induce long term differentiation of C2C12 myoblasts, but once again cells continued to proliferate on the patterns during this time, which made it very difficult to find patterns containing only a single cell.

Once again, we focused on the phosphorylation and nuclear translocation of SMAD1/5/8 after 4 h of culture, a hallmark of the early signaling associated to C2C12 osteogenic transdifferentiation (Fourel et al., 2016; Gilde et al., 2016). Using immunofluorescent staining of p-SMAD1/5/8, we examined SMAD1/5/8 phosphorylation and translocation to the nucleus, and how FN-bound BMP-2 affected this signaling pathway.

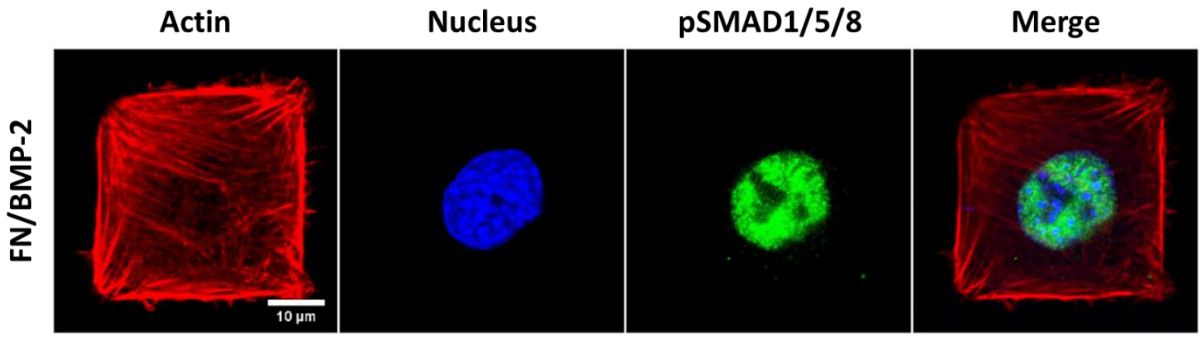


Figure 67: ***Micropatterned FN/BMP-2 induces SMAD signaling****. Immunofluorescence images of C2C12 myoblasts spread on small (500 µm²) and large (1500 µm²) micropatterns of FN and FN/BMP-2 after 4 hours of culture, showing no effect for FN, and nuclear enrichment of p-SMAD1/5/8 for FN/BMP-2. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green.*

As is shown in Figure 67, there was a clear enrichment in nuclear p-SMAD1/5/8 on FN/BMP-2 micropatterns, suggesting that BMP-2 mixed with FN and presented in a spatially controlled manner on (PLL/HA) films is able to trigger the activation of the SMAD signaling pathway in C2C12 myoblasts, and therefore their transdifferentiation toward an osteogenic lineage.

5. C2C12 myoblast adhesion and response to FN-bound BMP-2 micropatterns

a. Selective adhesion

Like patterns of BMP-2 alone, FN and FN/BMP-2 micropatterns on slightly crosslinked (PLL/HA) films are cell-adhesive areas on an otherwise non-cell-adhesive surface.

Indeed, as can be seen in Figure 68, C2C12 myoblasts selectively adhered on both the FN/BMP-2 and the FN patterns, whereas almost no cells were observed outside of the patterns.

Unlike on micropatterns of BMP-2 alone, we noticed that cells seemed to spread much better on patterns of FN and FN/BMP-2, both on 500 µm² and 1500 µm², and to fill the entire available pattern area.

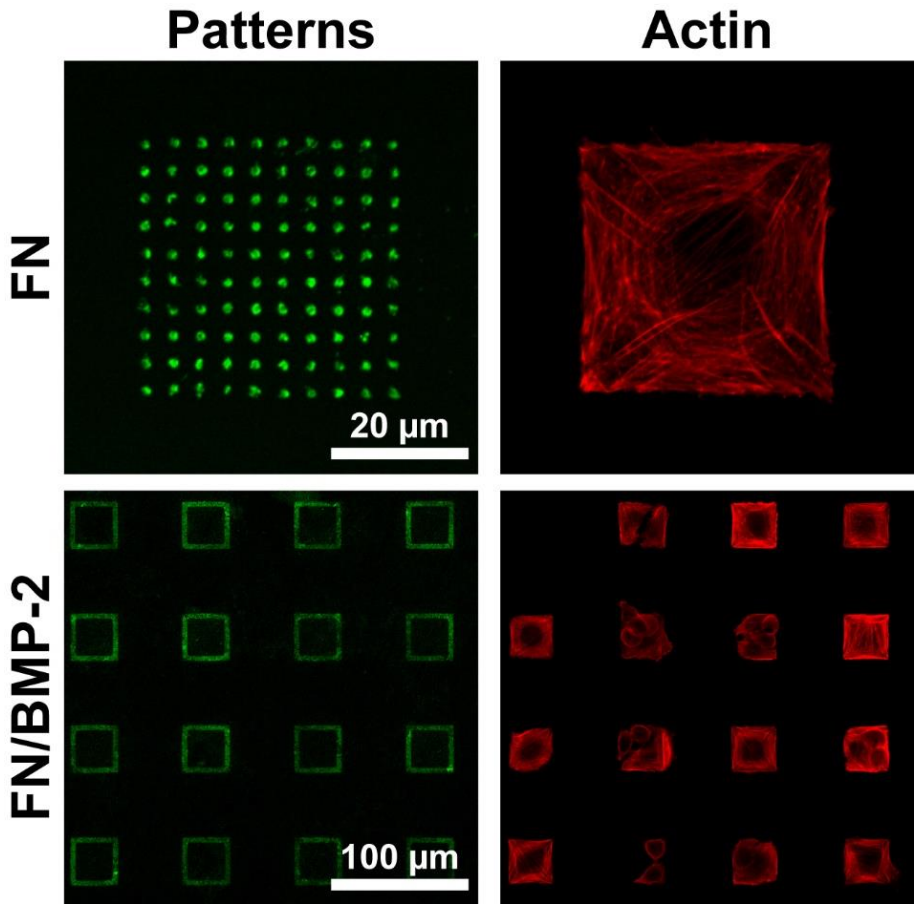


Figure 68: ***Selective adhesion of C2C12 myoblasts on the patterns****. Representative images of C2C12 myoblasts (actin staining in red) on FN and FN/BMP-2 patterns (in green), highlighting the very selective adhesion of the cells on the patterns, whereas no cells adhere outside of the patterns.*

b. Normalized cell spreading

We quantified the areas of a great number of patterns of FN and FN/BMP-2, and the areas of cells for all three conditions (*i.e.* cells spread on patterns of FN/BMP-2, and cells spread on patterns of FN with or without soluble BMP-2 (sBMP-2)). This was carried out for both 500 µm² and 1500 µm² square micropatterns.

To highlight the usefulness of patterning in the regulation of cell spreading, we also quantified the areas of cells spread on a cell adhesive (PLL/HA) film.

As can be seen in Figure 69, and contrary to what we had witnessed with patterns of BMP-2 alone, printing FN or FN/BMP-2 on (PLL/HA) films allowed us to control quite effectively cell spreading, for both small and large patterns.

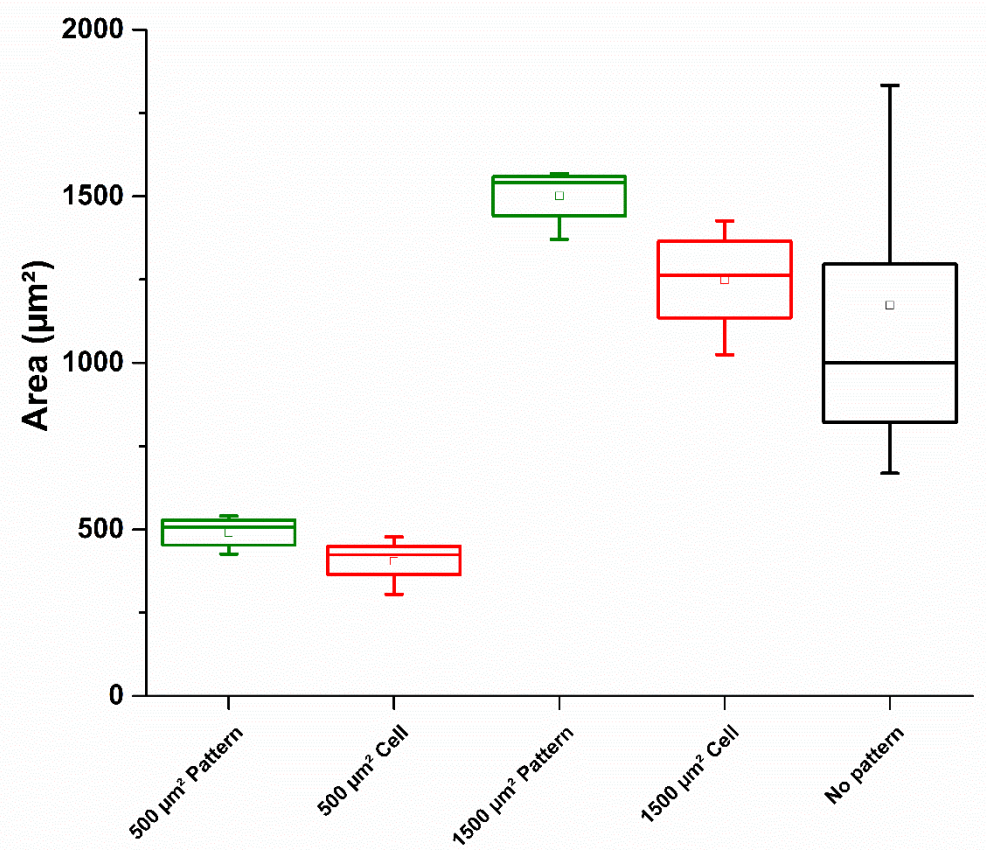


Figure 69: ***Pattern and Cell areas****. Quantification of pattern and C2C12 myoblast cell areas for 500 µm² and 1500 µm² square patterns of FN/BMP-2, and quantification of the cell areas of cells spread on a cell adhesive (PLL/HA) film.*

The areas measured are plotted in Figure 69 and summarized in Table 3.

Printed condition	FN/BMP-2 500 µm²	FN 500 µm²		FN/BMP-2 1500 µm²	FN 1500µm²		No pattern
		No BMP-2	+ sBMP-2		No BMP-2	+ sBMP-2	
Pattern area ± SD (µm²)	434 ± 28	447 ± 23		1502 ± 78	1350 ± 52		
# patterns	137	101		115	98		
Cell area ± SD (µm²)	405 ± 71	446 ± 35	487 ± 49	1249 ± 144	1320 ± 99	1389 ± 136	1094 ± 545
# cells	58	111	103	67	110	101	273

Table 3: ***Quantification of the effective micropattern size and corresponding cell areas****. Area of small and large square patterns, and of cells spread on them, for all three experimental conditions, i.e. FN/BMP-2, and FN with or without sBMP-2.*

Pattern and cell areas remain close, and the standard deviations are low, thus confirming that we are able to normalize cell spreading using patterns of FN/BMP-2, and FN with or without sBMP-2. Furthermore, the cell area on unpatterned (PLL/HA) highlights the extent to

which cell area can vary if uncontrolled, and therefore how difficult it is to assess the impact of spreading on other cellular behaviors without using patterning.

These cell areas also show the biological relevance of the pattern areas we chose to use in these works. Indeed, 500 µm² and 1500 µm² are roughly the average ± SD of C2C12 myoblasts when left to spread freely. This is consistent with values found in the literature (Crouzier, Fourel, et al., 2011). By choosing these pattern sizes, we are therefore forcing these cells to be either very round or highly spread out. Using the extremes of this specific cell type makes it easier to study specifically the impact of cell spreading on other biological processes.

c. Control of cytoskeletal tension

We have shown, using square micropatterns, that it is possible to use microcontact printing to regulate cell spreading on (PLL/HA) films. Nevertheless, it has also been shown that cytoskeleton tension plays a pivotal role in other cellular processes, such as stem cell fate determination (McBeath et al., 2004) and adhesion mechanics (Levy et al., 2010).

Considering how reproducible adhesion and spreading were on square patterns of FN/BMP-2, we wanted to see if it was possible to use microcontact printing to generate different cytoskeletal organizations, all the while maintaining a constant cell spreading.

To this end, we generated FN/BMP-2 patterns of various shapes, which we hypothesized would force cells to spread over the same area, but with different cytoskeletal tensions. These patterns, and representative images of cells spread on them, are shown in Figure 70.

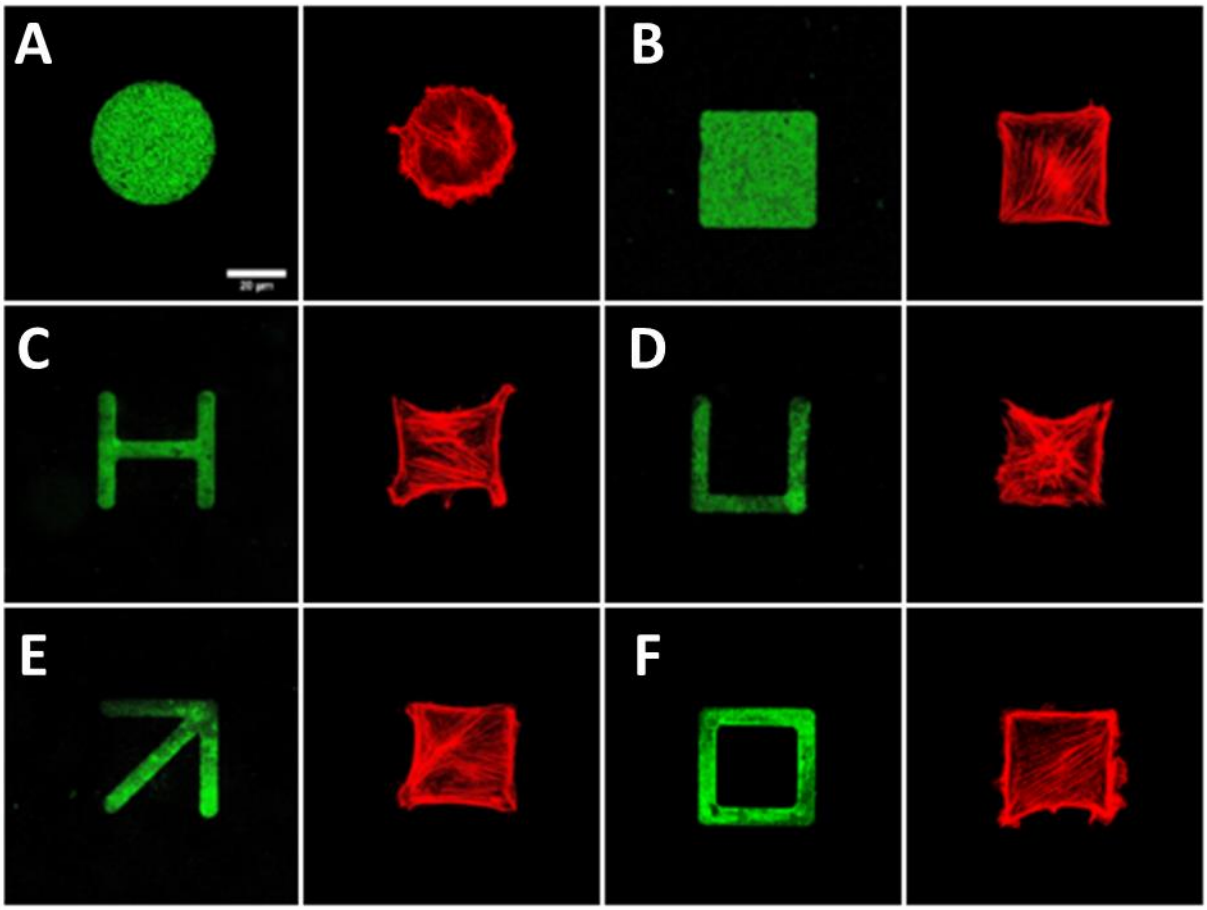


Figure 70: ***Using micropatterns to control cytoskeletal tension****. Representative images of patterns (in green) and cells (actin, in red), generated to create different cytoskeletal organizations in equally spread C2C12 myoblasts. (A) 1500 µm² Circle; (B) 1500 µm² Square; (C) H shape; (D) U shape; (E) Arrow shape; (F) 1500 µm² Hollow Square.*

On the representative images, we see that cells spread fully on these patterns, and maintained an overall square shape, regardless of the shape of the pattern. We can also see that cytoskeletal organization seems to be affected by these patterns (Figure 70).

Although we did not investigate further the effect of these differences in actin organization, these preliminary results show the potential usefulness of microcontact printing on (PLL/HA) films as a tool to control cell area and shape, and thus independently investigate the effect of cytoskeletal tension on cell behavior.

d. Subcellular processes

Similarly, we seeded C2C12 myoblasts on patterns comprised of a 10-by-10 array of small dots, spread evenly over a 1500 µm² square area (Figure 71 and Figure 72), to investigate the biological relevance of micrometer-range patterns.

We first observed the adhesion of single cells on such an array of printed FN/BMP-2. As is shown in Figure 71, not only does the cell spread out over the entire array, but the edges of the cell coincide with the outer edges on the protein array, thus demonstrating the cell's ability to recognize single dots in a 10-by-10 array.

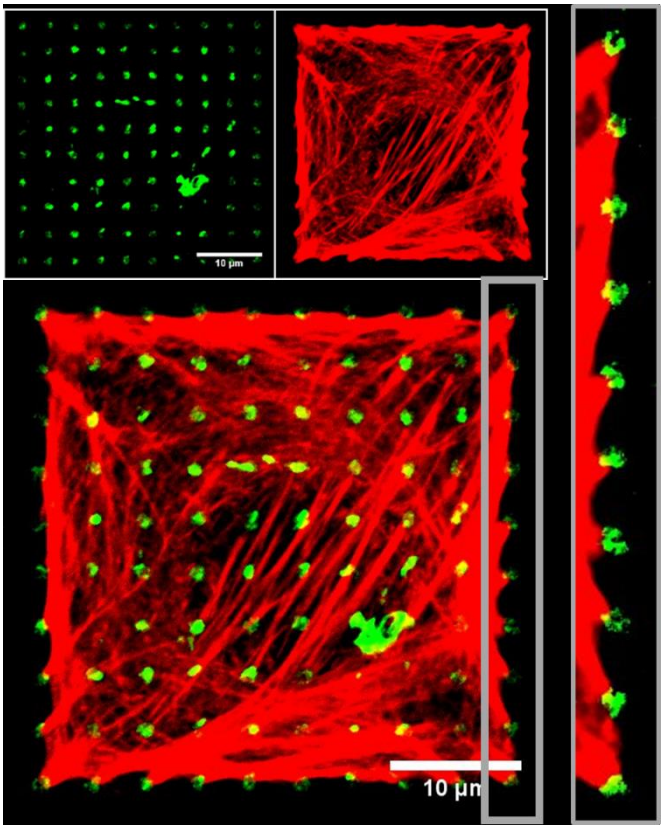


Figure 71: ***Single cell spread over a 1500 µm² array of 10-by-10****. Image of the FN/BMP-2^{CF} pattern, the actin cytoskeleton, and the merge of the two. On the right of the cell there is a zoom, showing how cells respond to these small dots by adhering specifically on them.*

Considering this observation, we hypothesized that focal adhesions were likely colocalized with the dots of the array, in particular on the outer edges of the cell. To test this hypothesis, we carried out an immunofluorescent staining of vinculin on C2C12 myoblasts spread on these patterns. Using confocal microscopy, we showed that vinculin, a membrane-

cytoskeletal protein involved in focal adhesions (FA), was confined on these micropatterns of ~3µm diameter (Figure 72).

This shows the ability of the cell to detect and respond to each dot of the array individually, and therefore the relevance of microcontact printing as a potential tool for the study of FAs.

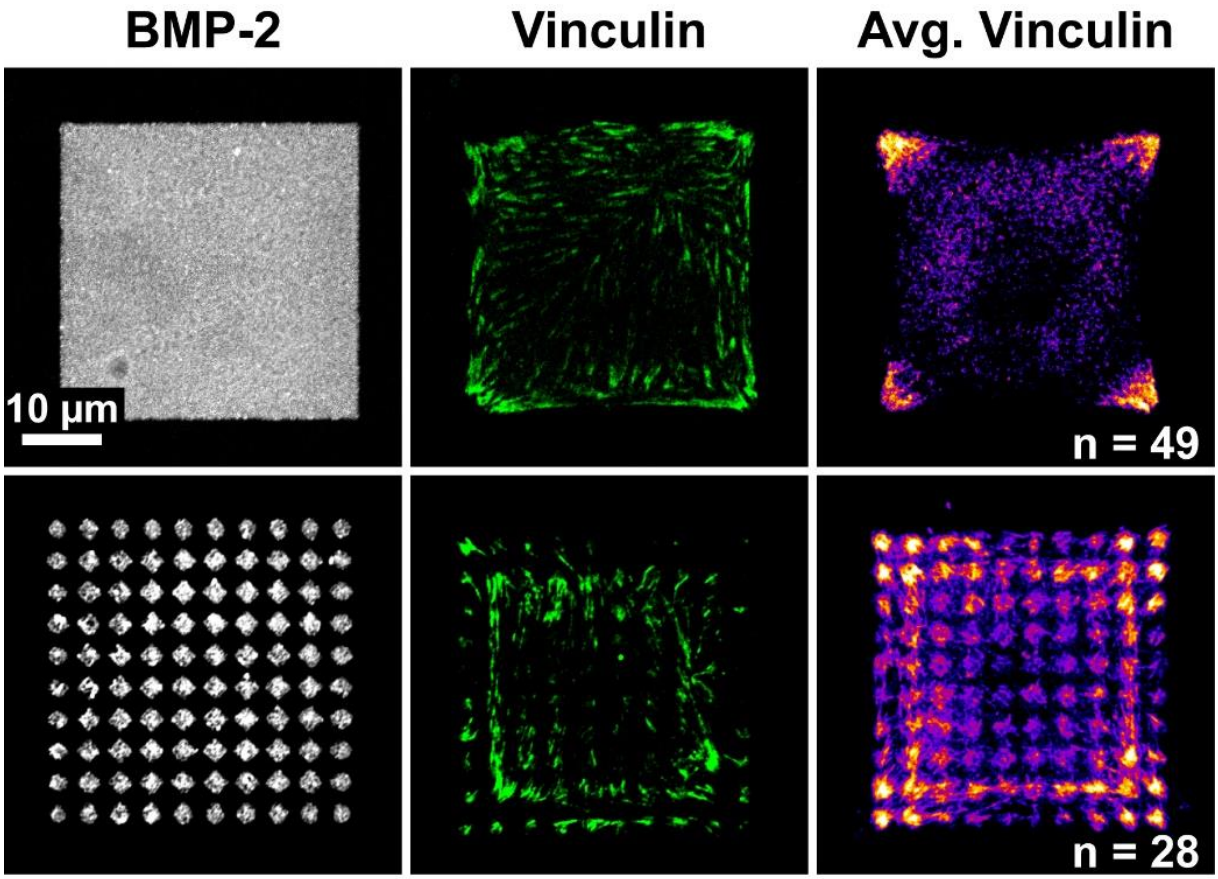


Figure 72: ***Focal adhesions in cellular and subcellular micropatterns****. Representative images of cellular (top row) and subcellular (bottom row, dot average diameter of 2.9 ± 0.3 µm) micropatterns of FN/BMP-2 (in gray), with corresponding representative immunostainings of vinculin and average vinculin images over n cells.*

Through these experiments, we were able to show that microcontact printing of FN/BMP-2 or FN alone is an efficient tool to induce a selective adhesion of C2C12 myoblasts on (PLL/HA) films, and to force these cells to spread fully over the entire area of the pattern. This allowed us to control the size of each cell, and therefore carry out experiments at a single cell level in highly normalized and reproducible conditions.

We were also able to control cytoskeletal tension independently of cell area, suggesting that these patterns can be used for studies attempting to study cellular tension without affecting cell spreading.

Finally, we were able to generate and show the biological relevance of subcellular patterns, which could prove to be a useful tool to study mechanics of adhesion, integrin or receptor recruitment, and even potential crosstalk between various cell surface receptors.

By associating microcontact printing and LbL deposition, we were thus able, for the first time, to generate cellular- and subcellular-sized micropatterns of unmodified BMP-2 trapped in FN on soft biopolymeric films without damaging the films. Due to the affinity between BMP-2 and FN (Fourel et al., 2016; Mikael M Martino et al., 2014; Mikaël M Martino & Hubbell, 2010), this presentation mode allowed us to potentiate the effect of BMP-2 and FN, in a manner close to *in vivo* conditions.

IV. Using micropatterns as a tool to answer complex biological questions

Previous work from our team has shown that cells on (PLL/HA) films respond to BMP-2, whether bound or in solution. Measuring cell area on these films also revealed that cell spreading was affected by BMP-2 presentation (Crouzier, Fourel, et al., 2011). As cell spreading can affect other aspects of cell behavior, we needed a tool to allow us to normalize cell spreading.

The results described above show that micropatterns on (PLL/HA) can accomplish this, and can thus be a versatile tool for the study of many cellular processes. Using this tool, we were able to investigate complex biological questions requiring the control of cell spreading.

1. Effects of BMP-2 presentation and cell spreading on cytoskeletal organization

One of the first biological questions we looked into was the effect of these patterns on cytoskeletal organization. Indeed, we observed noticeable differences in the actin

cytoskeleton of cells depending on the printing conditions. Furthermore, it had previously been shown that cell shape strongly regulates cell behaviors such as differentiation, mitosis or apoptosis (C. S. Chen et al., 1997; McBeath et al., 2004; Théry et al., 2005).

In order to evaluate the impact of cell spreading and cytoskeletal tension on the signaling pathways induced by FN-bound BMP-2, we generated, on (PLL/HA) films, small micropatterns (500 µm²) that roughly correspond to the size of attached but hardly spread C2C12 cells, and large micropatterns (1500 µm²) matching the size of fully spread C2C12 cells (Crouzier, Fourel, et al., 2011) (Figure 69).

Single C2C12 cells spread over the whole FN/BMP-2 or FN pattern and matched their shape, thus allowing us to control cell spreading and subsequently cytoskeletal organization and tension. C2C12 myoblasts presented a very specific cytoskeletal organization on the micropatterns, with very thick actin fibers along the sides of square patterns on FN and FN/BMP-2 patterns. Moreover, we observed that in the case of patterns of FN-bound BMP-2, there was a significant recruitment of actin fibers around the nucleus when compared to FN patterns with or without sBMP-2 (Figure 73).

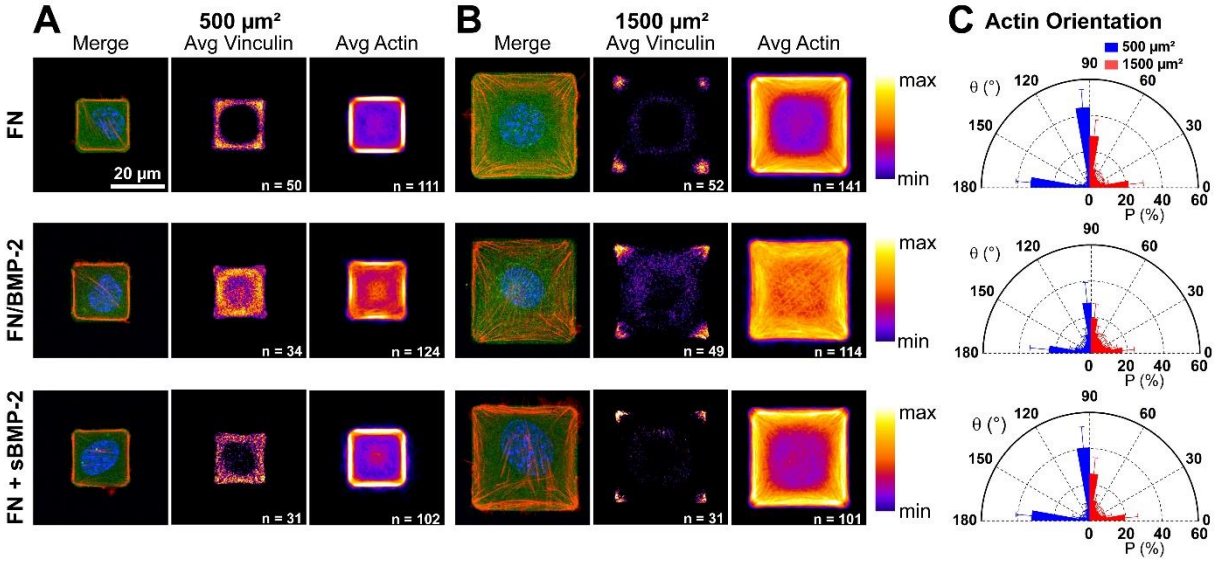


Figure 73: ***C2C12 myoblasts adhere and respond to micropattern geometry on soft biopolymeric films****. Individual C2C12 myoblasts, average vinculin and average actin images over n cells on small 500 µm² (A) and large 1500 µm² (B) micropatterns of FN/BMP-2 and FN alone with and without soluble BMP-2 (sBMP-2) after 4 h of culture. Micropatterns are in green, actin in red and nuclei in blue. (C) Corresponding actin orientation.*

We also performed immunofluorescent staining to observe the localization of vinculin. We noticed a good correlation between vinculin localization and actin organization (Figure 73A and B).

3D reconstruction of actin staining showed that C2C12 myoblasts on FN/BMP-2 patterns presented a strong and specific cytoskeletal continuity between peripheral stress fibers and the nucleus (Figure 74).

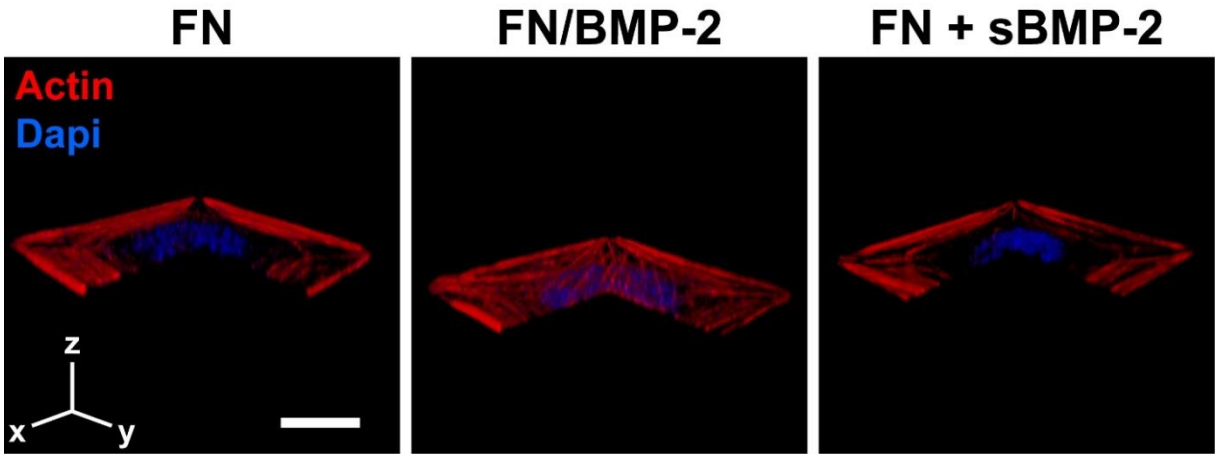


*Figure 74: **3D actin organization**. Representative 3D reconstructions of C2C12 myoblasts (actin in red, nucleus in blue) on 1500 µm² square micropatterns of FN/BMP-2 and FN without or with sBMP-2, highlighting the specific actin organization around the nucleus only for cells on micropatterns containing BMP-2. Scale bar is 10µm.*

Indeed, vinculin was more prominent on the outer edge of the FN patterns, in the presence or not of sBMP-2, whereas it was more homogeneously located over the entire area of the cell on patterns of FN/BMP-2.

The quantification of actin orientation (Figure 73C) revealed that about 75 % of actin fibers were parallel to the square sides (*i.e.* with an angle < 10° or > 80°) on 500 µm² FN squares, and 50% aligned along the sides of 1500 µm² FN squares without sBMP-2. Actin organization was not affected by the presence of sBMP-2 and presented similar orientations, confirming the importance of BMP-2 presentation mode on cell behavior, and in particular actin organization.

For all conditions, the differences in relative actin orientation observed between the 500 and the 1500 µm² squares is mainly due to the presence of radial actin stress fibers on the larger patterns, which come and reinforce the actin cytoskeleton to sustain their large spreading

(Bereiter-Hahn, Lück, Miebach, Stelzer, & Vöth, 1990; Mandal, Balland, & Bureau, 2012). We found less aligned actin fibers on FN/BMP-2 micropatterns, with percentages decreasing to 50% and 36% on small and large squares, respectively. This loss of actin alignment on BMP-2-containing patterns is mostly due to the emergence of numerous reinforcing fibers around the nucleus (Figure 73 and Figure 74).

This organization closely resembled what can be observed on patterns of BMP-2 alone, although the fact that cells did not fully spread on 1500 µm² BMP-2 patterns made the interpretation of the average actin maps very complicated and unreliable.

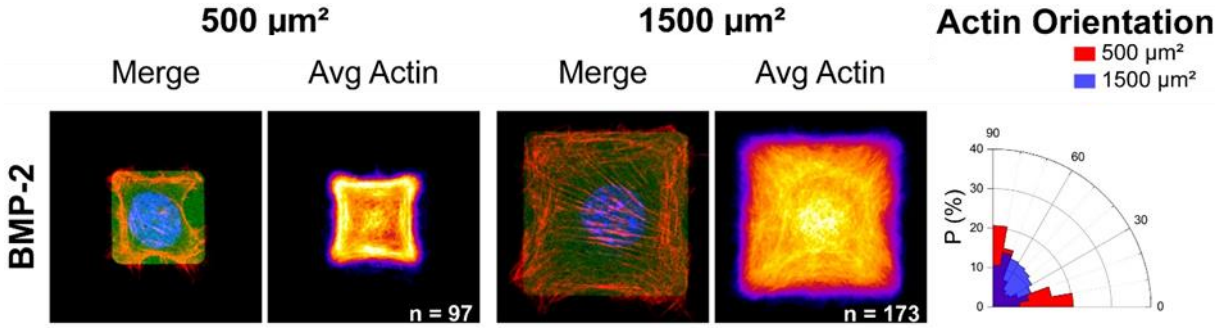


Figure 75: ***C2C12 myoblasts adhere and respond to micropattern geometry on soft biopolymeric films****. Individual C2C12 myoblasts, average actin images over n cells and actin orientation on small 500 µm² and large 1500 µm² micropatterns of BMP-2, after 4 hours of culture. Micropatterns are in green, actin in red and nuclei in blue.*

Indeed, as can be seen in Figure 75, C2C12 myoblasts presented a very similar cytoskeletal organization on BMP-2 micropatterns, with similar thick actin fibers along the sides of square BMP-2 patterns. On 500 µm² BMP-2 patterns, 70 % of actin fibers were parallel to the square sides (*i.e.* with an angle < 20° or > 70°).

On large 1500 µm² squares, 40% of actin fibers were aligned along the sides of BMP-2 patterns. Similarly, this loss of actin alignment on larger patterns is mostly due to the emergence of numerous reinforcing fibers along the square diagonals (*i.e.* around 45°) and around the nucleus. This actin reinforcement is particularly striking on the 3D reconstruction shown in Figure 76.

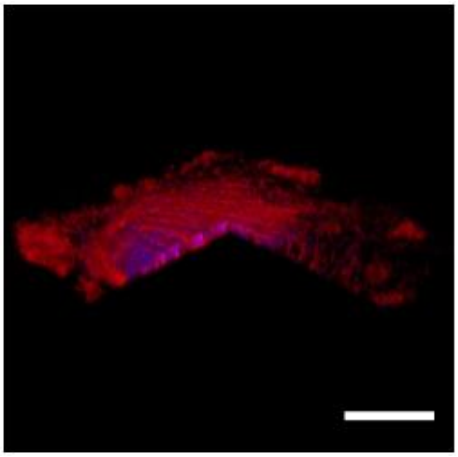


Figure 76: ***3D actin organization****. Representative 3D reconstruction of C2C12 myoblasts (actin in red, nucleus in blue) on a 1500 µm² square micropattern BMP-2 alone. Scale bar is 10µm.*

These results strongly support the idea that this reorganization of the actin cytoskeleton surrounding the nucleus is truly brought about by the matrix-bound presentation of BMP-2.

Thanks to the contrast the the cell-adhesive properties of the slightly crosslinked (PLL/HA) films and the FN, we could simultaneously control the spatial presentation of FN-bound BMP-2 and cell spreading, revealing the specific impact of this presentation mode of BMP-2 on the cytoskeletal continuity between peripheral stress fibers and the nucleus of C2C12 myoblasts.

As we observed a clear impact of FN-bound BMP-2 on cytoskeletal organization, especially around the nucleus, we next investigated whether this effect was correlated with BMP-2-dependent regulation of transcription factors.

2. FN-bound BMP-2-induced SMAD signaling depends on cell spreading

To investigate the effect of cell spreading on early osteogenic transdifferentiation, we once again used immunofluorescent staining to observe the phosphorylation and nuclear translocation of SMAD1/5/8. We used confocal microscopy and image analysis to measure this nuclear enrichment, in order to have a quantitative measure of the effect of BMP-2, its presentation mode, and cell spreading on this signaling pathway.

First we verified the specificity of the immunostaining of p-SMAD1/5/8 by examining its presence and location in C2C12 myoblasts spread on control FN patterns, without (negative) or with BMP-2 in solution (positive). Representative images of these cells, and the quantification of nuclear p-SMAD1/5/8 enrichment are shown in Figure 77.

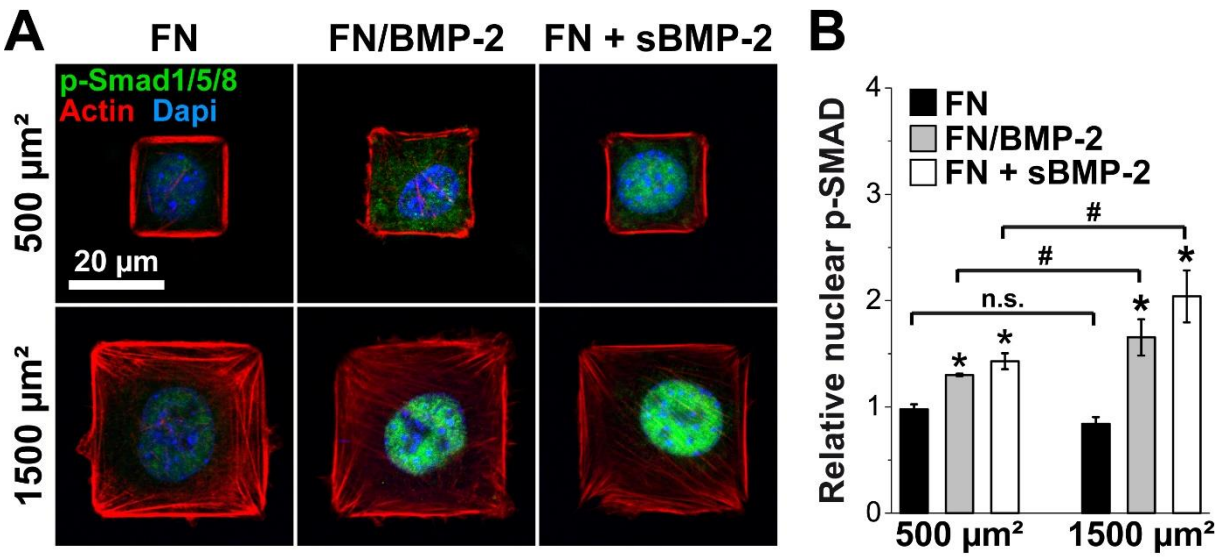


Figure 77: ***Phosphorylation and translocation of SMAD1/5/8 to the nucleus depends on cell spreading****. (A) Immunofluorescence images of C2C12 myoblasts spread on small (500 µm²) and large (1500 µm²) micropatterns of FN/BMP-2 and FN without (negative control) or with (positive control) BMP-2 in solution after 4h of culture. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green. (B) Quantification of the relative nuclear p-SMAD1/5/8 (n > 100 cells) in function of the size and composition of the micropatterns. * p < 0.01 versus negative control (FN patterns without BMP-2 in solution); # p< 0.01 between small and large micropatterns. n.s. stands for non-significant (i.e. p > 0.01).*

In the absence of BMP-2, we observed only a small amount of p-SMAD1/5/8 in the cytoplasm and nucleus of C2C12 myoblasts plated on both small and large FN micropatterns. In the presence of BMP-2 in solution, p-SMAD1/5/8 was highly enriched in the nuclei of myoblasts adhering on FN patterns. Furthermore, this amount of nuclear p-SMAD1/5/8 appeared to depend on cell area, increasing with the degree of cell spreading (Figure 77B).

The presentation of FN-bound BMP-2 also triggered the phosphorylation of SMAD1/5/8 and its translocation into the nucleus. Moreover, we observed similar effects of cell spreading on the phosphorylation of SMAD1/5/8 (Figure 77). The level of nuclear p-SMAD1/5/8 was significantly higher for myoblasts spread over large, 1500µm² patterns (1.89 ± 0.58) compared to smaller, 500 µm² squares (1.28 ± 0.21).

Once again, similar changes in nuclear p-SMAD1/5/8 enrichment could be observed on patterns of BMP-2 alone (Figure 78), with a significant increase between small and large micropatterns (from 1.14 ± 0.29 to 1.47 ± 0.27).

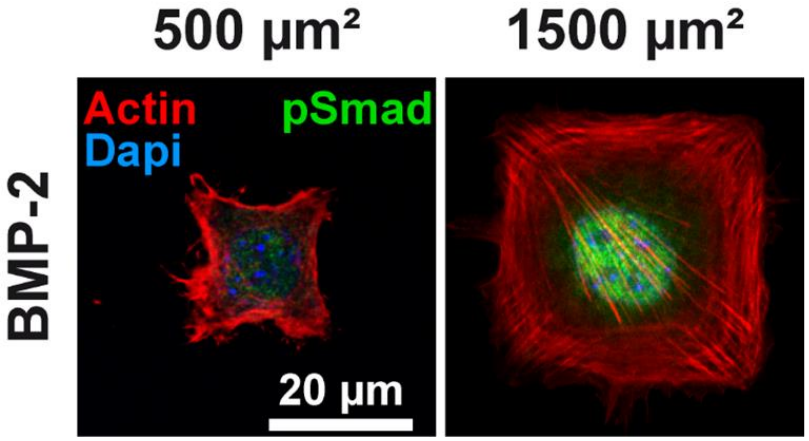


Figure 78: ***Phosphorylation of SMAD1/5/8 and translocation to the nucleus depends on cell spreading****. Left: Immunofluorescence images of C2C12 myoblasts spread on small (500 µm²) and large (1500 µm²) micropatterns of BMP-2 after 4 hours of culture. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green. Right: Quantification of the normalized ratio of nuclear over cytoplasmic p-SMAD1/5/8 in function of the size of the micropatterns. # p< 0.01 between small and large micropatterns.*

We thus demonstrated that a matrix-bound presentation of BMP-2 was able to trigger the phosphorylation of SMAD1/5/8 and its translocation to the nucleus. For the first time, we demonstrated that BMP-2-induced osteogenic transdifferentiation signaling was also regulated by cell spreading when BMP-2 was bound to FN.

Of note, we observed a similar adaptation of the cell morphology to the pattern for two other cell types, namely mouse mesenchymal stem cells (D1 MSCs) and human immortalized myoblasts (hMyoblasts) (Figure 79).

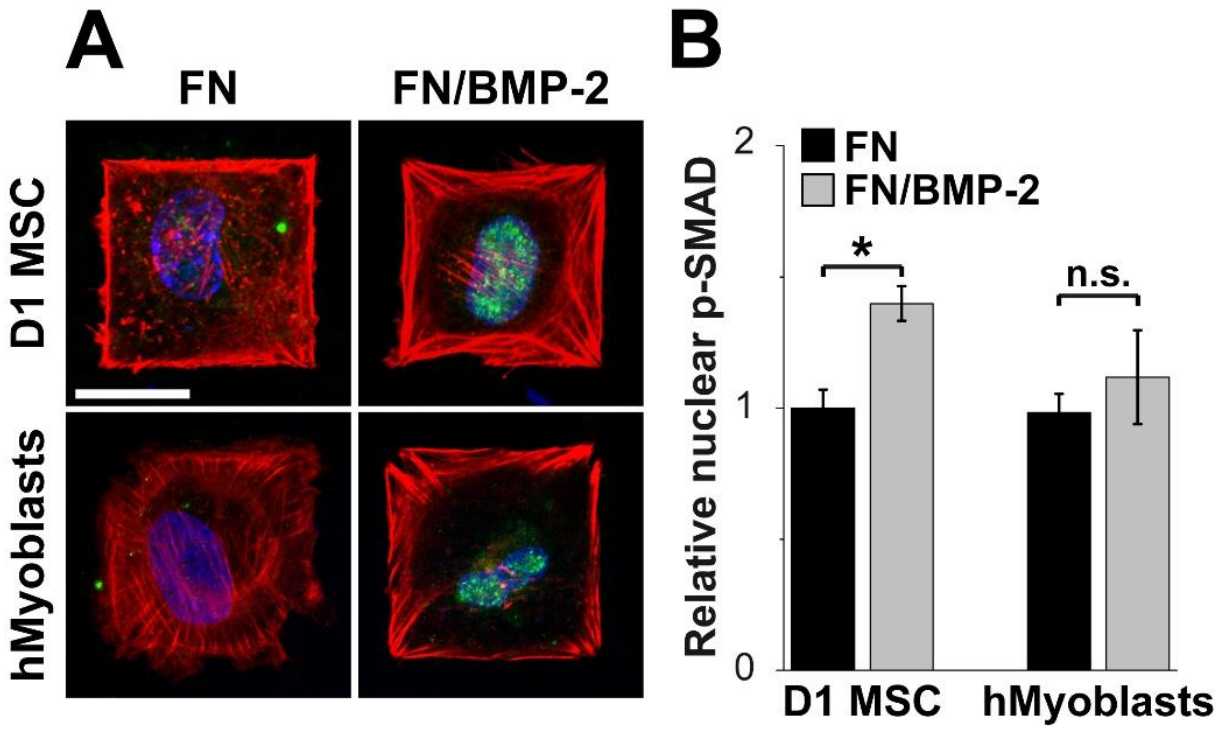


Figure 79: ***Phosphorylation and translocation of SMAD1/5/8 to the nucleus of D1 mesenchymal stem cells and human myoblasts****. (A) Immunofluorescence images of D1 mesenchymal stem cells (D1 MSC) and human myoblasts (hMyoblasts) spread on large (1500 µm²) micropatterns of FN alone (negative control) and FN/BMP-2 after 4h of culture. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green. (B) Quantification of the relative nuclear p-SMAD1/5/8 in function of the composition of the micropatterns for D1 MSC (n > 60 cells) and hMyoblasts (n > 20 cells). * p < 0.01 versus negative control (FN patterns without BMP-2 in solution); n.s. stands for non-significant (i.e. p > 0.01).*

We observed an elevated amount of nuclear p-SMAD1/5/8 for both cell types, which was statistically significant only for D1 MSCs (Figure 79B). Knowing that we are able to control the shape of D1 murine MSCs using micropatterns could make them an interesting research model to study how cell shape can regulate MSC fate determination, for example by looking at the impact of circle versus square patterns on adipogenesis versus osteogenesis.

We observed that the impact of BMP-2 was much lower in hMyoblasts. We hypothesize that this is likely due to the fact that even large 1500 µm² micropatterns were not large enough to accommodate these cells.

On FN/BMP-2 micropatterns, the amount of BMP-2 presented to the cell is proportional to the pattern size. Thus, in order to confirm that the increase in phosphorylation and nuclear translocation of SMAD1/5/8 when cells spread on large versus small BMP-2 patterns was indeed due to cell spreading, and not to the available amount of BMP-2, we compared the levels of p-SMAD1/5/8 in cells spread on solid versus hollow squares of FN/BMP-2 (Figure 80). The available spreading area is the same for both conditions, even though the amount of available BMP-2 is lower on the hollow squares (about 1/3 of the BMP-2 amount of solid square patterns).

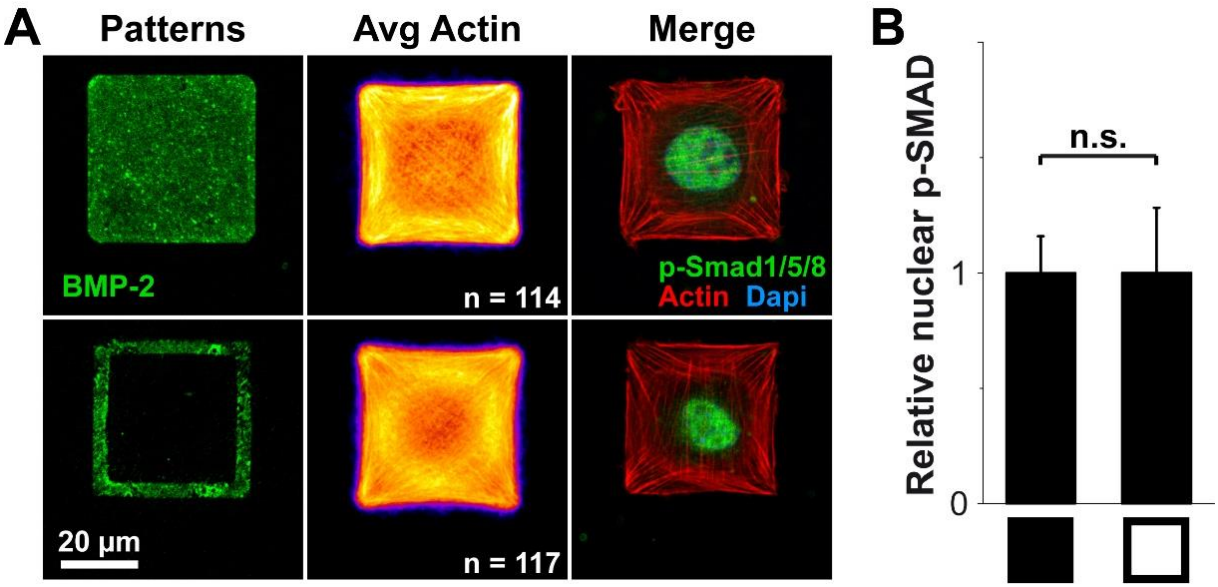


Figure 80: ***Spreading-dependent BMP-2-induced p-SMAD1/5/8 translocation is independent of the available amount of BMP-2****. (A) Solid (top) and hollow (bottom) 1500 µm² square micropatterns of FN/BMP-2 with BMP-2 in green, corresponding average actin images over n cells and immunofluorescence images of C2C12 myoblasts after 4h of culture where actin is in red, nuclei in blue and p-SMAD1/5/8 in green. (B) Quantification of the relative nuclear p-SMAD1/5/8 for both conditions (n > 100 cells). n.s. stands for non-significant (i.e. p > 0.01).*

Cells spread across the non-printed areas on hollow squares and presented similar spreading area and cytoskeleton organization as cells on solid patterns. The amount of nuclear p-SMAD1/5/8 was similar for both conditions (Figure 80). Thus, cell spreading appears to be determinant in regulating BMP-2-induced SMAD1/5/8 signaling.

Of note, during preliminary experiments, we also generated 1000 µm² micropatterns of FN/BMP-2 and FN with or without sBMP-2, as an intermediate condition between poorly (500 µm²) and highly spread out (1500 µm²) cells.

Cells spread on these patterns displayed intermediate behaviors, both in terms of actin organization and p-SMAD1/5/8 signaling (Figure 81). As these results do not really help furthering our understanding of the studied processes we chose to focus solely on patterns of 500 µm² and 1500 µm² in these works.

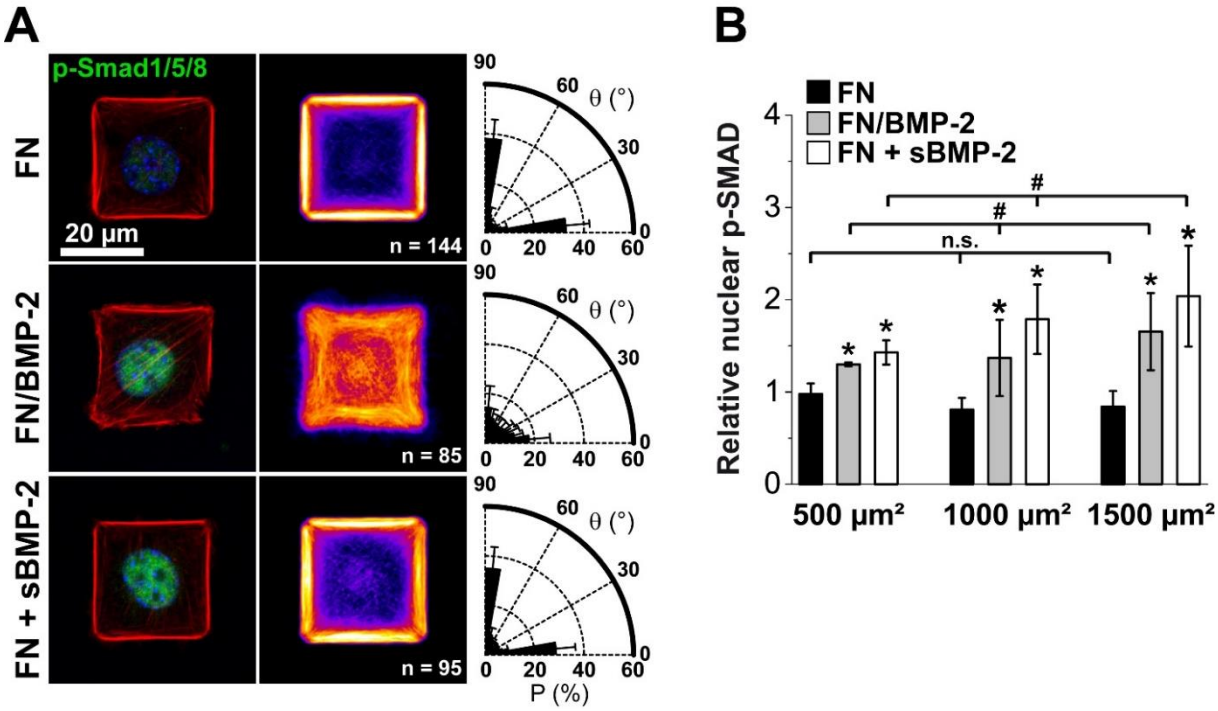


Figure 81: ***Response of C2C12 myoblasts on intermediate 1000µm² micropatterns*** *(A) Individual C2C12 myoblasts, average actin images over n cells and corresponding actin orientation on intermediate 1000 µm² micropatterns of FN/BMP-2 and FN alone with and without sBMP-2 after 4 h of culture. Actin is in red, nuclei in blue and p-SMAD1/5/8 in green. (B) Quantification of the relative nuclear p-SMAD1/5/8 (n > 80 cells) in function of the size and composition of the micropatterns. * p < 0.01 versus negative control (FN patterns without BMP-2 in solution); # p < 0.01 between small, intermediate and large micropatterns. n.s. stands for non-significant (i.e. p > 0.01).*

3. SMAD signaling is induced by FN-bound BMP-2 through a LIMK-dependent pathway

To further our understanding of the potential role of the cytoskeleton in FN-bound BMP-2-induced SMAD1/5/8 signaling, we used a pharmacological and siRNA-mediated knockdown approach to interfere with cell tension and cytoskeleton dynamics (Figure 82), and

subsequently with BMP-2-mediated SMAD signaling (Fourel et al., 2016; Y.-K. Wang et al., 2012). Indeed, teams have identified an involvement of the RhoA and ROCK pathways in BMP-2-induced SMAD signaling and the osteoblastic differentiation of human MSCs (Y.-K. Wang et al., 2012). Furthermore, work carried out by some of our collaborators has shown that LIMK-dependent actin dynamics contribute to SMAD signaling induced by BMP-2 bound on a soft matrix (Fourel et al., 2016). These studies guided the choice of pharmacological inhibitors and siRNAs we used in the works described below.

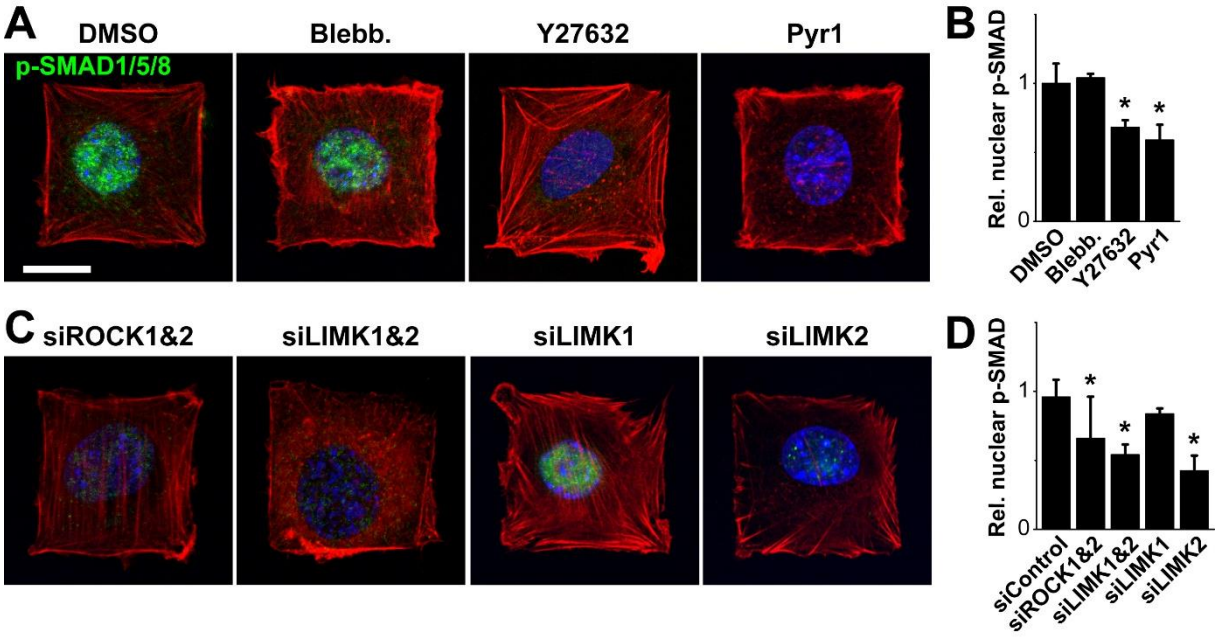


Figure 82: ***Early SMAD1/5/8 signaling induced by FN-bound BMP-2 depends on LIM kinase but not myosin II activity.*** *(A) Immunofluorescence images and (B) quantification of the relative nuclear p-SMAD1/5/8 (n > 60 cells) of C2C12 myoblasts after 4h of culture on 1500 µm² square micropatterns of FN/BMP-2 in presence of DMSO, blebbistatin (Blebb), Y27632 or Pyr1. (C) Immunofluorescence images and (D) quantification of the relative nuclear p-SMAD1/5/8 (n > 60 cells) of C2C12 myoblasts depleted in ROCK1&2, LIMK1&2, LIMK1 or LIMK2 using a siRNA strategy. Actin is in red, nuclei in blue, and p-SMAD1/5/8 in green. Scale bar is 20µm. * p < 0.01 versus control (i.e. DMSO or siControl).*

Cell shape and spreading were unaffected by the inhibition of the motor protein myosin II by blebbistatin (Straight et al., 2011), the inhibition of the Rho-associated kinase (ROCK) by Y27632 (Uehata et al., 1997) or the inhibition of the ROCK effector LIM kinase (LIMK), which inhibits the actin-depolymerizing protein cofilin, by Pyr1 (Prudent et al., 2012) (Figure 82A). The amount of nuclear p-SMAD1/5/8 was unchanged by the inhibition of myosin II, whereas the inhibition of ROCK and of LIMK induced a drastic and significant decrease of nuclear p-SMAD1/5/8 (Figure 82B).

The siRNA-mediated silencing of ROCK1&2 and LIMK1&2 confirmed the effects of the chemical inhibitors (Figure 82C), both of them inducing a statistically significant decrease of nuclear p-SMAD1/5/8 (Figure 82D).

As only LIMK2 is a downstream effector of ROCK (Riento & Ridley, 2003), we used specific siRNA knockdowns against LIMK1 and LIMK2 and observed a strong decrease of the level of nuclear p-SMAD1/5/8 only when LIMK2 was silenced, confirming the crucial role of the ROCK-LIMK2 pathway in the BMP-2-induced early SMAD signaling (Figure 82C and D). The efficiency of siRNA-mediated knockdown was confirmed by Western blots (Figure 83).

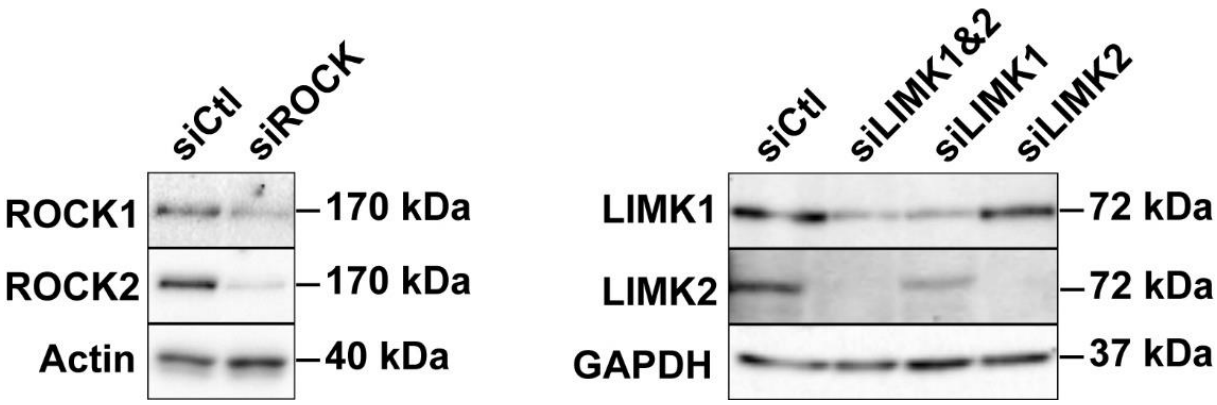


Figure 83: ***Efficiency of siRNA-mediated knockdown of ROCK1&2 and LIMK1&2.*** *Western blot analysis confirms the efficiency of the siRNA against ROCK1&2, LIMK1&2, LIMK1 and LIMK2.*

Of note, we obtained similar results with cells on FN patterns with sBMP-2, with no effect of blebbistatin on the nuclear p-SMAD1/5/8 and a strong decrease with Y-27632, Pyr1, siROCK1&2 and siLIMK1&2 (Figure 84).

These results suggest that the cytoplasmic phosphorylation and nuclear translocation of SMAD1/5/8 in C2C12 cells on FN/BMP-2 patterns is mediated by ROCK and LIMK2, in a myosin II-independent manner.

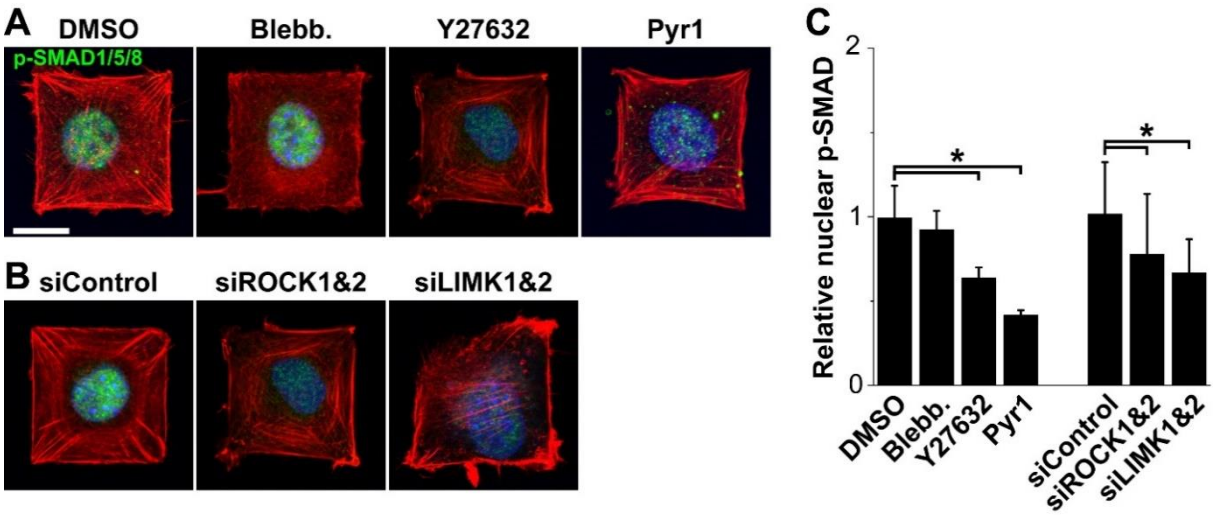


Figure 84: ***Early SMAD1/5/8 signaling induced by soluble BMP-2 depends on LIM kinase but not on myosin II.*** *(A) Immunofluorescence images of C2C12 myoblasts after 4 h of culture on 1500 µm² square micropatterns of FN with sBMP-2 in presence of DMSO (control), blebbistatin (Blebb), Y27632 or Pyr1. Actin is in red, nuclei in blue, and p-SMAD1/5/8 in green. (B) Immunofluorescence images of C2C12 myoblasts after siRNA-mediated knockdown in ROCK1&2 or LIMK1&2 using siRNA strategy. (C) Quantification of the relative nuclear p-SMAD1/5/8 (n > 60 cells). Scale bar is 20µm. * p < 0.01 versus control (i.e. DMSO or siControl).*

V. Preliminary results

1. Adhesion and spreading mechanism

a. Adhesion and spreading kinetics

In addition to the differences in terms of cytoskeletal organization and SMAD signaling which we have previously described on patterns of FN/BMP-2 and FN with or without sBMP-2, we also noticed differences in adhesion and spreading kinetics which appeared to be linked to the presentation mode of BMP-2.

Indeed, when imaging C2C12 myoblasts spread on these patterns after 30 minutes of culture, we noticed that cells on FN/BMP-2 patterns were already fully adhered and spread. On patterns of FN with or without sBMP-2, however, cells were barely starting to adhere, and appeared round and poorly spread out (Figure 85).

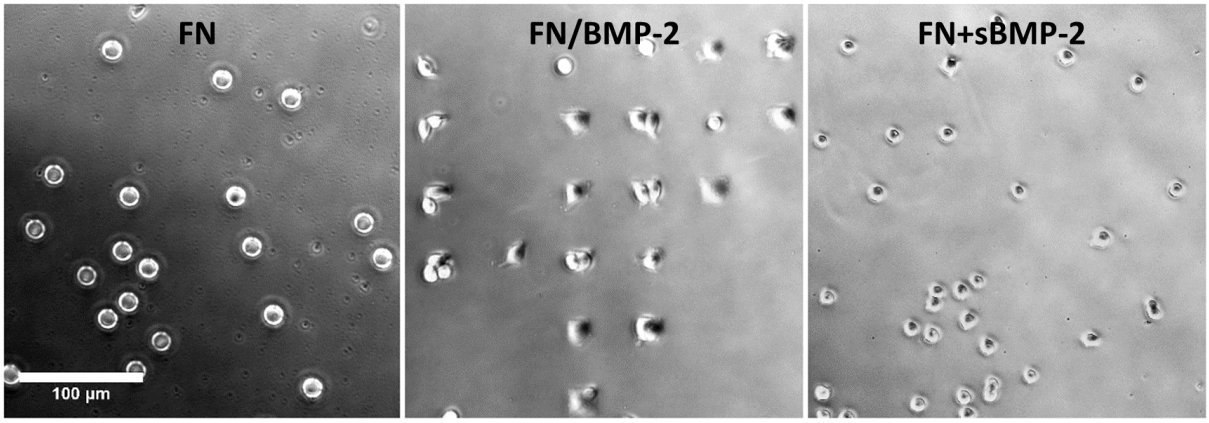


Figure 85: ***Adhesion kinetics depending on printing conditions****. Representative brightfield images of C2C12 myoblasts on micropatterns of FN/BMP-2 and FN with or without sBMP-2, 30 minutes after seeding, highlighting important differences in adhesion and spreading kinetics.*

These results suggest a pivotal role of BMP-2 presentation mode on adhesion and early spreading mechanisms, and deserve to be studied in more depth.

The advantage of using micropatterns to study cell spreading is that it is very easy to see if cells are spread, and to what extent, and also to measure their spreading kinetics, knowing that maximum cell spreading is limited by the pattern area. Future works would therefore involve studying the early stages of cell adhesion and spreading, and quantifying the speed at which cells adhere and spread fully on these patterns.

b. Involvement of integrins in cell adhesion

Considering these differences in spreading kinetics, we posit that different printing conditions might lead to the recruitment of different integrins, and also that these differences may be due to the existence of crosstalk between integrins and BMP-2 receptors. Indeed, it was recently shown that there is a crosstalk between β3 integrins and BMPR-Ia receptors when matrix-bound BMP-2 is presented to C2C12 cells in a homogeneous manner (Fourel et al., 2016).

As a preliminary experiment to investigate this possibility, we used siRNA interference to silence the expression of integrins β1, β3 and β5, on 1500 µm² square micropatterns of

FN/BMP-2. Using qPCR, we checked the effectiveness of the siRNA strategy for each silenced integrin Table 4.

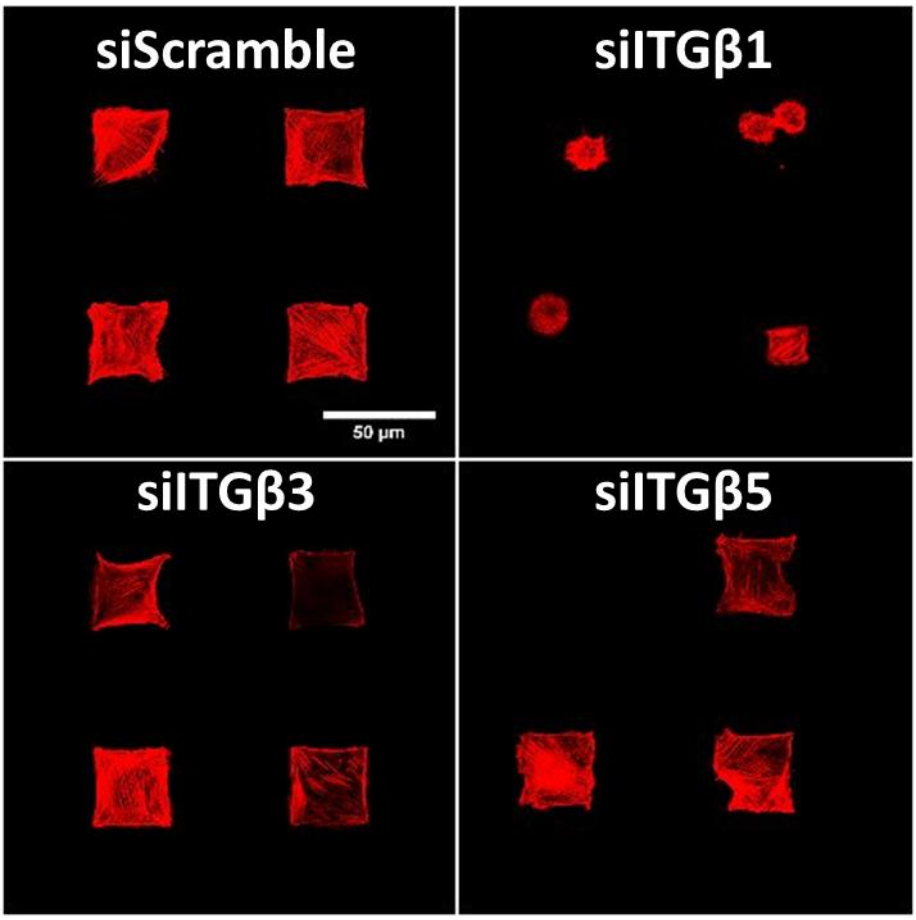


Figure 86: ***Effect of integrin silencing on cell spreading on FN/BMP-2 micropatterns****. Representative images of C2C12 cells spread on square 1500 µm² patterns after 4 hours of culture and specific gene silencing using a siRNA strategy.*

siRNA	Relative decrease in expression
ITGB1	98%
ITGB3	82%
ITGB5	98%

Table 4: ***Quantification of the relative decrease in gene expression for each silenced integrin, highlighting the effectiveness of the siRNA strategy****.*

As we can see in Figure 86, the silencing of integrins β3 or β5 in C2C12 cells on these micropatterns did not affect cell spreading after 4 hours, as cells were still able to fully spread and fill the entire pattern area. When integrin β1 was silenced, however, cells successfully adhered but were completely unable to spread on the pattern.

These results suggest a crucial role of integrin β1 in the ability of C2C12 myoblasts to spread on micropatterns of FN/BMP-2. These experiments need to be repeated to confirm these findings, and could be pursued with the silencing of various combinations of integrins, as a tool to investigate potential compensation mechanisms between different integrin subunits. Associating this siRNA strategy with nuclear p-SMAD1/5/8 quantification may also reveal which integrins are involved in SMAD signaling on these patterns.

2. Cytoskeleton organization and nuclear positioning

We have shown that C2C12 myoblasts spread on square micropatterns of FN/BMP-2 and FN with or without sBMP-2 on (PLL/HA) films present very different cytoskeletal organizations, with strong fibers of actin reinforcing the cytoskeleton diagonally, and clear actin presence surrounding the nucleus (Figure 73 and Figure 74).

We also previously demonstrated the ability of these micropatterns to control cell shape and induce SMAD signaling in D1 murine MSCs (Figure 79).

To further investigate this cytoskeletal reorganization, and how it relates to printing conditions, we seeded D1 cells on micropatterns of FN/BMP-2 and FN with or without BMP-2, printed on PDMS. We used PDMS rather than (PLL/HA) films for these experiments, as it allowed us to have a more clear fluorescent signal, with less background noise, and therefore a more precise measurement of fluorescent intensity throughout the cell. We then imaged a vertical cross-section of the cell over its entire height in z, to see if the presence or presentation mode of BMP-2 affected the actin cytoskeleton or the nucleus.

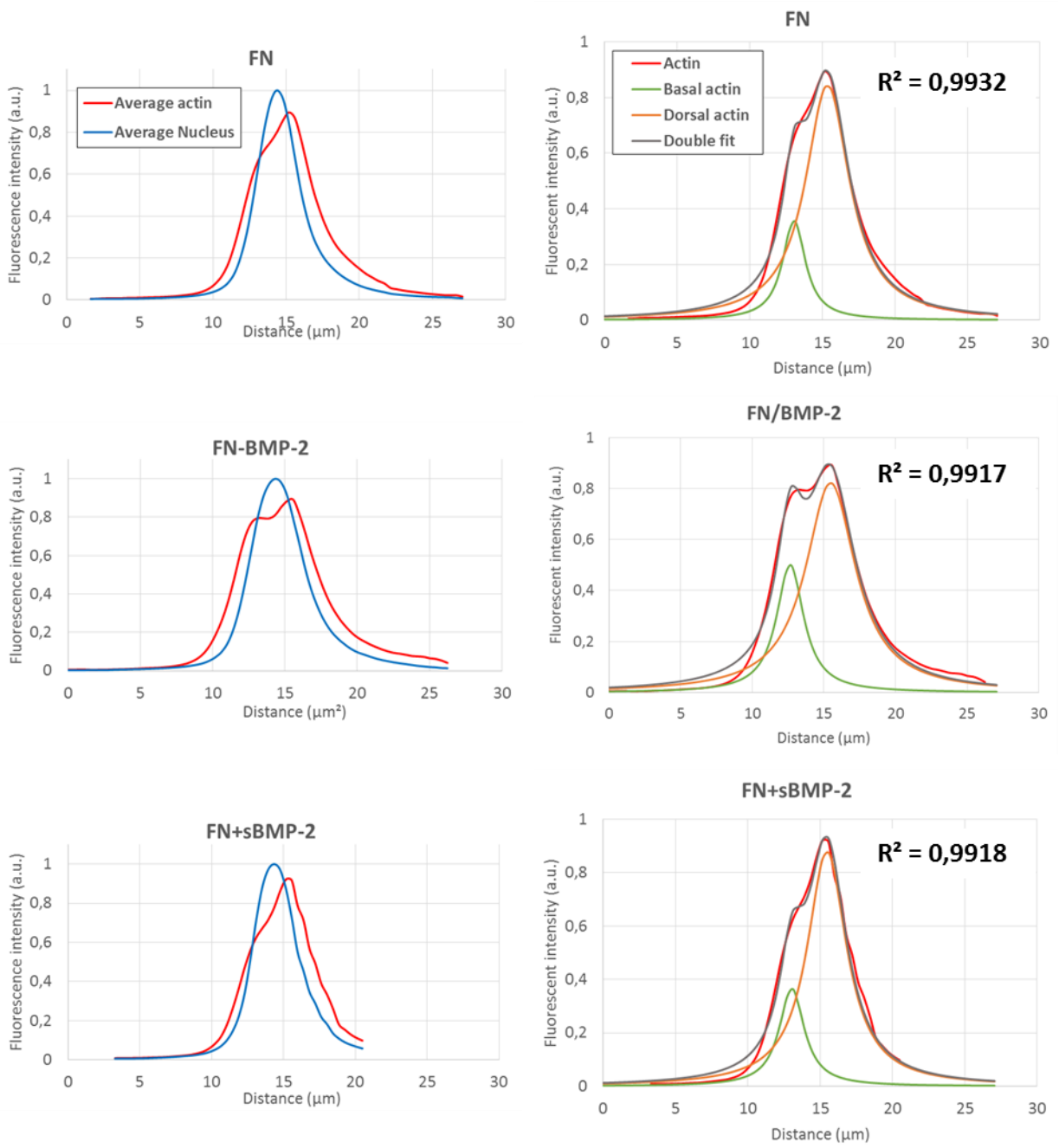


Figure 87: ***BMP-2 presentation mode strongly affects cytoskeletal organization and nuclear positioning****. Normalized fluorescence intensity of actin (in red) and nucleus (in blue), in the z direction, in D1 murine MSCs spread on 1500 µm² square patterns of FN/BMP-2 (n=19) and FN with (n=21) or without (n=23) sBMP-2, highlighting the effect of BMP-2 presentation mode on cytoskeletal organization surrounding the nucleus.*

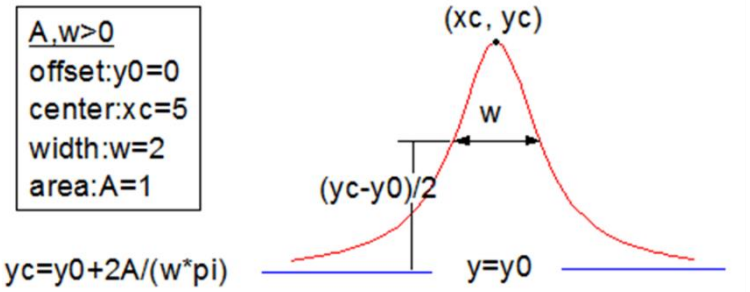
As shown in Figure 87, there is no noticeable difference in cytoskeletal organization surrounding the nucleus in D1 cells on patterns of FN with or without sBMP-2. When we present BMP-2 bound to FN, however, we see a clear change in actin around the nucleus. Indeed, there seems to be a strong presence of actin both below and above the nucleus, as

demonstrated by the presence of two peaks in the actin signal on either side of the peak in nucleus signal, and suggesting that the nucleus is maintained within a cage of actin.

To verify this observation, we used a double-Lorentzian function (Equation 1) and a least-squares method to fit the fluorescent intensity of actin for all three conditions (Figure 87, right). Using this function, we were able to obtain a good fit for all three profiles, and identify the two separate peaks due to the presence of actin below and above the nucleus.

We chose a Lorentzian rather than a Gaussian fitting function due to the quality of the fit, in particular relating to the tail end of the function.

$$y = y_0 + \frac{2A}{\pi}\frac{w}{4(x - x_c)^2 + w^2}$$



Equation 1 : ***Equation of a single Lorentzian function***

The parameters used for each fit are displayed in Table 5, as well as the ratio of basal to dorsal actin peaks. These results show that there is no difference between patterns of FN with or without sBMP-2, whereas a FN-bound presentation mode of BMP-2 induces a much more important presence of basal actin. This is demonstrated by the important changes in basal versus dorsal peak ratios depending on the printing condition. This ratio is calculated using the value of yc shown in Equation 1.

FN				FN/BMP-2				FN+sBMP-2			
	Basal actin	Dorsal actin	Basal/dorsal peaks (%)		Basal actin	Dorsal actin	Basal/dorsal peaks (%)		Basal actin	Dorsal actin	Basal/dorsal peaks (%)
y0	0	0	42,3	y0	0	0	60,9	y0	0	0	41,6
A	1,1	4,9		A	1,8	5,5		A	1,2	4,6	
w	1,9	3,7		w	2,3	4,3		w	2,1	3,3	
xc	13,0	15,4		xc	12,6	15,5		xc	13,0	15,5	

Table 5: ***Summary of the fitting parameters and peak ratio for patterns of FN/BMP-2 and FN with or with sBMP-2.***

As we had previously observed, the peak which corresponds to the actin below the nucleus was much more pronounced for patterns of FN/BMP-2 than FN with or without sBMP-2.

These results confirm that BMP-2 presentation mode has an effect on cytoskeletal organization around the nucleus. Keeping in mind the differences in vinculin organization described in Figure 73, it would be interesting to see how the mechanisms underlying this phenomenon, from focal adhesions to the nucleus via the actin cytoskeleton, interact with each other and affect other cellular behaviors like transcription factors or gene expressions.

3. Cell presence induces matrix remodeling on patterns of FN/BMP-2

We have previously shown that micropatterns printed on (PLL/HA) remain stable over time. While cells maintain their overall shape and size, we did however notice some changes in pattern aspect during this time, but only on patterns with cells spread on them.

Indeed, prior to cell seeding on micropatterns of FN/BMP-2, the protein always appears to be organized in a very homogeneous manner, with no preferential direction over the entire surface of the pattern. This homogeneous aspect remains true regardless of the shape or aspect ratio of the pattern (Figure 88).

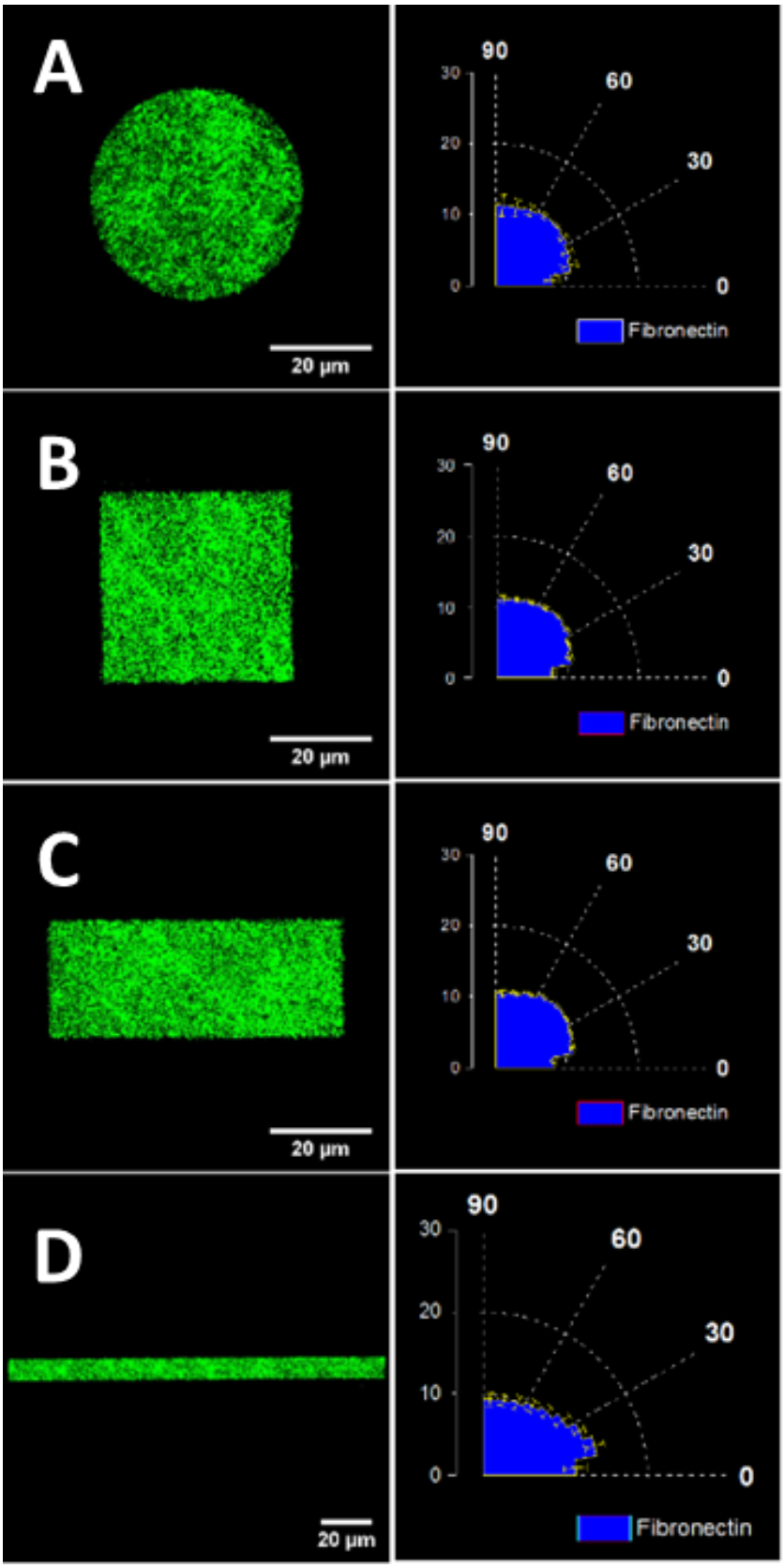


Figure 88: ***FN organization in patterns of increasing aspect ratio, prior to cell seeding****. Representative image of a FN/BMP-2 pattern (FN in green) and fibronectin orientation, on 1500 µm² (A) circular, (B) short band, (C) square and (D) long band patterns.*

On patterns after several days of cell culture, however, we noticed that the pattern was modified by the presence of the cell, and that fibronectin was reorganized. To further investigate this, we cultured C2C12 myoblasts on patterns of the same area (1500 µm²) but of different aspect ratios for 4 days, and looked at fibronectin organization after this time. We chose these patterns because they present a drastic change in cell shape, meaning that differences in cytoskeletal organization and their effect on fibronectin remodeling would be all the more visible.

As can be seen in Figure 89, there were indeed very marked differences in actin orientation between cells spread on these different patterns. There is no preferential actin organization in cells spread on circular patterns, whereas on square patterns we once again see fibers close to 0 and 90°, following the edges of the pattern, and some diagonal fibers reinforcing the cytoskeleton. For patterns of higher aspect ratio (hereafter named short and long band), the preferential orientation in the length of the pattern was evident, with most actin fibers presenting an orientation close to 0°.

As we had anticipated, when we measured the orientation of fibronectin fibers on these patterns, we observed a strong correlation with actin orientation. Indeed, for all patterns the fibronectin was strongly remodeled in the same direction as the cytoskeleton. We posit that these changes are linked to cytoskeletal tension, and to the forces exerted by the cell on its underlying matrix, which in turn result in its modification.

These results reflect the two-way aspect of cell-matrix communication. Indeed, up until now, we had only studied ways in which we could impact cell behavior by changing the matrix, through the size, shape, or biochemical composition of the pattern. Here however, we see that by exerting force on the pattern, the cell is also able to remodel and change it over time, which is an indication of inside-out signaling in these cells, potentially mediated by integrins. Silencing the expression of integrins or focal adhesion proteins, or disrupting actin dynamics, may therefore affect matrix remodeling, and could be used to investigate how each link in the chain connecting the ECM to the actin cytoskeleton intervenes in this pathway. These patterns presenting modified FN organization could also be used as a tool to

study how an already organized FN matrix can impact certain cell behaviors, and in particular differentiation.

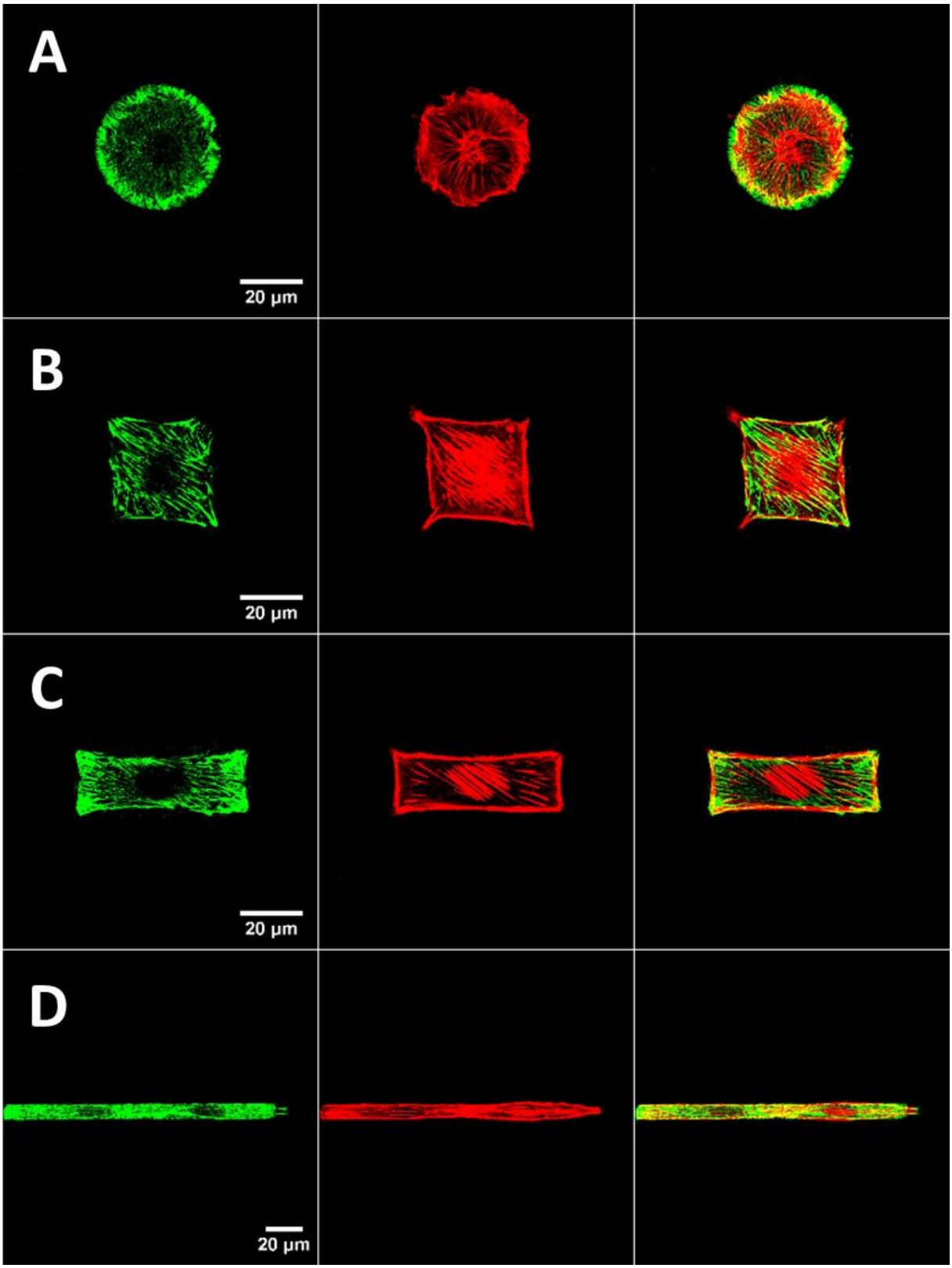


Figure 89: ***FN directionality in patterns of increasing aspect ratio, after 4 days of culture****. Representative image of a FN/BMP-2 pattern (FN, in green), cytoskeleton (actin, in red), merge, and fibronectin (blue) and actin (red) orientations, on 1500 µm² (A) circular, (B) square, (C) short band and (D) long band patterns.*

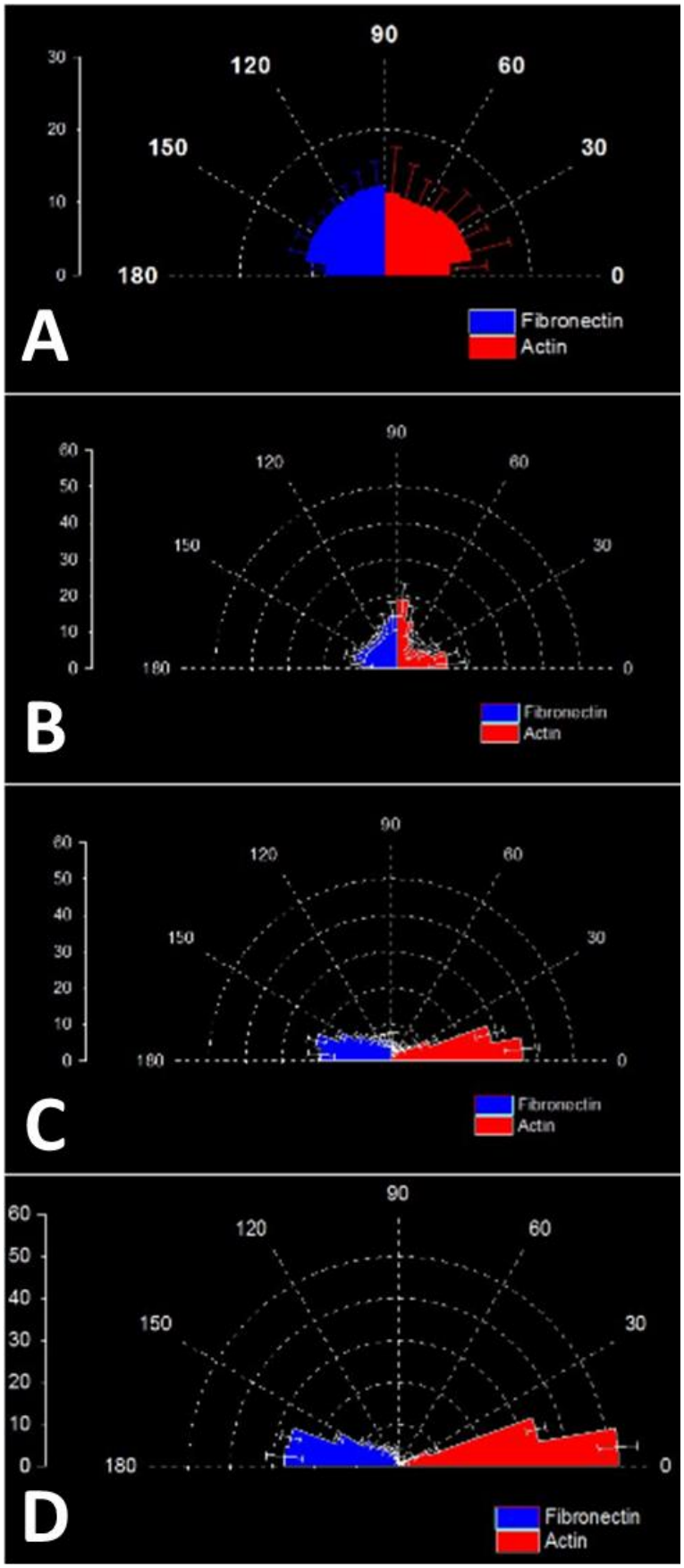


Figure 90: ***Cell shape affects fibronectin remodeling in FN/BMP-2 micropatterns****. Fibronectin and actin orientations on 1500 µm² (A) circular, (B) square, (C) short band and (D) long band patterns.*

4. BMP-2 presence and presentation mode affect cellular force

The results described thus far suggest that cells are able to detect biochemical changes in their underlying matrix and respond to them by adjusting their focal adhesions, cytoskeletal organization and transcription factors, but also by exerting forces on their microenvironment.

To investigate the effect of BMP-2 presentation on cellular forces, we carried out traction force experiments on C2C12 myoblasts spread on polyacrylamide gels (E = 14kPa) containing fluorescent beads, and homogeneously printed with FN/BMP-2 and FN with or without sBMP-2.

After 4 hours of culture, we selected six cells for each printing condition and imaged the fluorescent beads. We then lysed the cells using sodium dodecyl sulfate, and once again imaged the fluorescent beads in the same field. Using homemade Matlab protocols, this allowed us to measure the contractile energy of the cells depending on the printing condition. The results of these measurements are displayed in Figure 91.

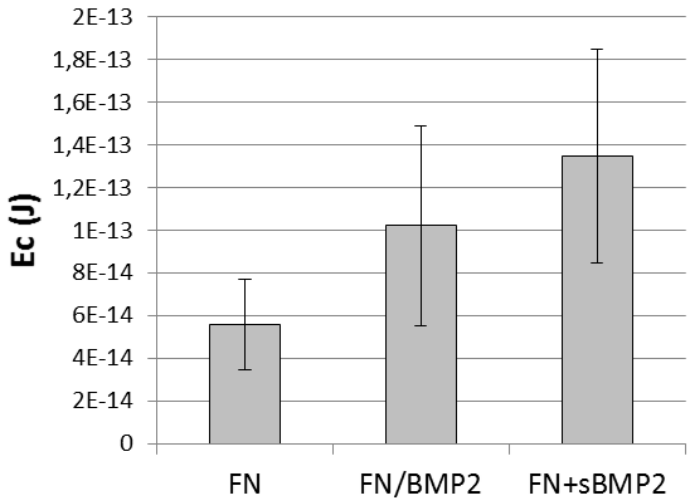


Figure 91: ***BMP-2 presence and presentation mode affect cellular force****. Contractile energy (in J) of cells spread on homogeneously patterned gels of polyacrylamide (E=14kPa), highlighting the effect of the presence of BMP-2 and its presentation mode on cellular forces.*

The contractile energy of the cells increased in the presence of BMP-2, whether matrix-bound or in solution. This contractile energy was even more pronounced when BMP-2 was

presented in solution. This trend strongly resembles the evolution of relative nuclear p-SMAD1/5/8 for the same printing conditions, on 1500 µm² square patterns and on (PLL/HA) films (Figure 77).

While preliminary, these results thus suggest a link between BMP-2 presentation, cellular forces, and SMAD signaling. Future experiments based on these data would investigate how these forces lead to a nuclear translocation of p-SMAD1/5/8, through mechanotransduction from the ECM to the nucleus.

DISCUSSION

BMP-2 is a very potent morphogen playing an important role in morphogenesis and tissue homeostasis (Hogan, 1996; Kopf et al., 2014), and appears to be a powerful inducer of osteogenic differentiation in MSCs (Reddi, 1998; Y.-K. Wang et al., 2012), osteoblasts (Reddi, 1998; Wall & Board, 2014) and myoblasts (Katagiri et al., 1994).

Interestingly, although the mode of presentation of BMP-2 (*i.e.* in solution or matrix-bound) has been shown to distinctly regulate cellular responses (Crouzier, Fourel, et al., 2011; Wei et al., 2015), the impact of a biomimetic, FN-bound presentation of BMP-2 on cell adhesion, cytoskeletal organization and BMP-2 signaling is still mostly unknown.

We now show that the degree of cell spreading, as well as the presentation of BMP-2 by an ECM protein, can regulate actin organization and BMP-induced SMAD signaling, and in the case of FN does so through a LIM kinase activation. Given the key role of BMP-2 in developmental processes, the link between BMP-2 presentation mode, cytoskeletal organization and BMP-2 signaling may participate in specific osteogenic differentiation events during musculo-skeletal development. Moreover, understanding how matrix-bound BMP-2 regulates osteogenic differentiation is crucial in the development of clinical therapies for critical bone defects (Nisarg J Shah et al., 2014; Shekaran et al., 2014).

I. Selective cellular adhesion on micropatterned substrates

Taking advantage of the very good affinity between BMP-2 and FN (Mikael M Martino et al., 2014; Mikaël M Martino & Hubbell, 2010), we generated micropatterns of BMP-2 within FN on soft biopolymeric films. The potential of these LbL films loaded with BMP-2 for implant coatings was previously demonstrated (Crouzier, Sailhan, et al., 2011; R. Guillot et al., 2016; N. J. Shah et al., 2013; Nisarg J Shah et al., 2014). (PLL/HA) films were chosen as the ability of cells to adhere and spread on these films strongly depends on the film's crosslinking (Boudou et al., 2011; Richert et al., 2004) and/or the presence of matrix-bound BMP-2 (Crouzier, Fourel, et al., 2011; Fourel et al., 2016).

Slightly crosslinked (PLL/HA) films were thus previously shown to have lasting and stable non-cell-adhesive properties (Boudou et al., 2011; Richert et al., 2004), while cells adhere and rapidly spread on the same films containing matrix-bound BMP-2 (Crouzier, Fourel, et al., 2011; Fourel et al., 2016). By microcontact printing patterns of FN/BMP-2 on these films, we thus generated cell-adhesive micropatterns on a non-cell-adhesive surface, with a subcellular spatial resolution, close to 3 µm (Figure 65), which is comparable to resolutions obtained for microcontact printing on stiff substrates (Théry & Piel, 2009).

Moreover, we avoided any protein modification, which could affect the biological activity of the BMP-2 (Alborzinia et al., 2013; Y. Ito et al., 1998; Nakaji-Hirabayashi et al., 2007). The films were not damaged during the microcontact printing and BMP-2 was immobilized within a mesh of FN strands **(**Figure 63 and Figure 64). When we seeded C2C12 myoblasts on micropatterned films, we observed a very selective adhesion of the cells only on the micropatterns, with almost no cell outside of the patterns (Figure 68).

II. Cytoskeletal organization is impacted by controlled cell spreading

Previously, it had been shown that cell shape strongly regulates cell behaviors such as differentiation, mitosis or apoptosis (C. S. Chen et al., 1997; McBeath et al., 2004; Théry et al., 2005). By designing small and large micropatterns that roughly correspond to the size of unspread C2C12 cells and fully spread C2C12 cells, respectively (Crouzier, Fourel, et al., 2011), we could control and normalize cell spreading and, subsequently, cytoskeletal organization and tension (Figure 69 and Figure 70).

C2C12 myoblasts presented a very specific cytoskeletal organization on the micropatterns, with very thick actin fibers along the sides of square patterns on FN and FN/BMP-2 patterns. The presence of FN-bound BMP-2 but not sBMP-2 induced a recruitment of actin fibers localized around the nucleus, suggesting that the presence of BMP-2 within the FN, or the resulting proximity of BMP receptors and integrins (Fourel et al., 2016), regulated the cytoskeleton organization quite distinctly from free BMP-2 in solution.

III. SMAD signaling depends on BMP-2 presentation and cell spreading

We demonstrated that BMP-2 mingled with FN was bioactive, as it triggered a significant translocation of p-SMAD1/5/8 into the nucleus (Figure 67) and ALP activity (Figure 66). We showed that the amount of nuclear p-SMAD1/5/8 increased with the degree of cell spreading for both conditions of soluble and FN-bound BMP-2 (Figure 77).

Our results are consistent with those obtained by Wang *et al.* for human MSCs cultured on stiff substrates in the presence of soluble BMP-2 (Y.-K. Wang et al., 2012). But contrary to this previous work, the available amount of BMP-2 in our study depends on the area of the micropatterns when BMP-2 is presented by the matrix. By comparing the levels of p-SMAD1/5/8 in cells spread on solid versus hollow squares of FN/BMP-2, we demonstrated that cell spreading was determinant when BMP-2 was bound to the FN (Figure 80).

IV. SMAD signaling is mediated by a LIMK-dependent and myosin II-independent pathway

Recently, the presentation mode of BMP-2 was shown to influence cell behavior, as matrix-bound BMP-2 (Crouzier, Fourel, et al., 2011) and covalently grafted BMP-2 (Wei et al., 2015) were found to trigger stronger and more sustained effects than soluble BMP-2. Wang *et al.* found that SMAD signaling, induced by soluble BMP-2 in human MSCs cultured on fibronectin-coated plates, was regulated by cytoskeletal tension via the ROCK pathway (Y.-K. Wang et al., 2012). In contrast, Fourel *et al.* demonstrated using soft biopolymeric films and C2C12 cells that early SMAD signaling was dependent on the Cdc42/LIMK pathway and independent of ROCK (Fourel et al., 2016). Using micropatterns of FN-bound BMP-2 on a soft biopolymeric film, we showed that both ROCK and LIMK2 are involved in SMAD signaling, in a myosin II–independent manner (Figure 82). A summary of the signaling pathways investigated in these works is shown in Figure 92.

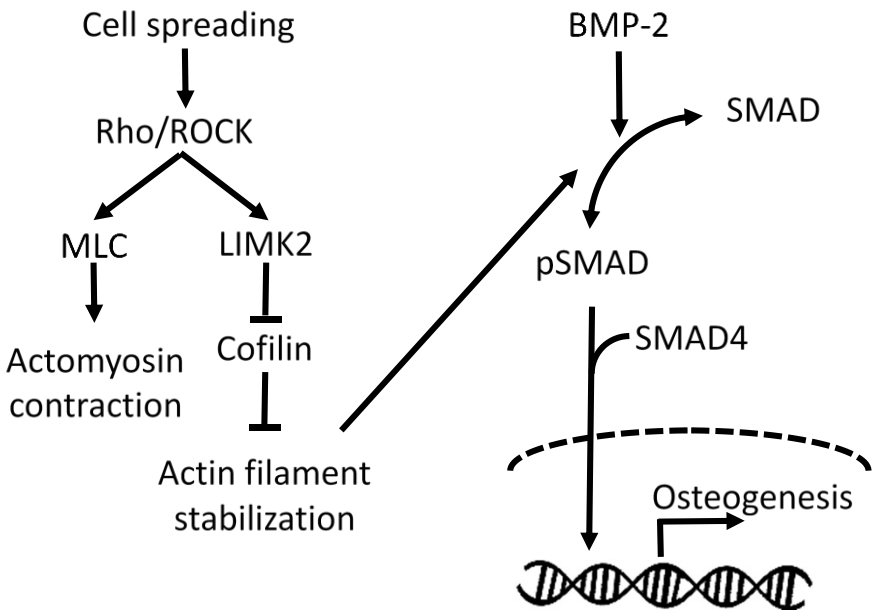


Figure 92: ***Signaling pathways investigated in the present study****. Cell spreading, through the activation of the RhoA/ROCK pathway, was shown to impact the cytoplasmic phosphorylation and nuclear translocation of SMAD1/5/8. This regulation was carried out in a LIMK-dependent and MLC-independent manner. It was therefore mediated by changes in actin filament stabilization, enacted by cofilin, and not actomyosin contraction.*

These slight discrepancies may be due to differences in experimental settings such as cell type (starved human MSCs (Y.-K. Wang et al., 2012) or non-starved C2C12 myoblasts (Fourel et al., 2016)), BMP-2 presentation mode, underlying substrate (fibronectin-coated plate (Y.-K. Wang et al., 2012) or soft biopolymeric film (Fourel et al., 2016)) or even the source of FN (provided by the cells (Fourel et al., 2016), microcontact printed alone (Y.-K. Wang et al., 2012) or mingled with BMP-2 (in the present study).

As our study focused on early SMAD signaling after 4 hours of culture, we do not exclude the involvement of myosin II in FN-bound BMP-2-induced signaling at later stages of differentiation. Nevertheless, the role of LIMK in the phosphorylation and inactivation of cofilin supports the need for spatial and temporal control of actin organization to initiate or maintain the osteogenic commitment (Sen et al., 2015). Since BMP-2 plays a ubiquitous role in regulating morphogenesis, this link between BMP-2 presentation mode, cellular mechanics and signal transduction may provide a broader model for better understanding the impact of the spatial presentation of growth factors by ECM proteins on tissue formation, organization and homeostasis.

V. Signaling mechanisms, from the extracellular matrix to the nucleus

The results we have described up to this point, whether in terms of actin cytoskeleton organization, vinculin distribution or SMAD signaling, raised several questions regarding the signaling mechanisms and pathways at play in cells spread on our patterns.

Indeed, we were able to create spatially defined matrices for cells to spread on, and to induce nuclear translocation of p-SMAD1/5/8 using these matrices, but wished to investigate further how cells communicated with this imposed ECM, and the mechanisms at play in the mechanotransduction of cytoskeletal tension from the cell membrane to the nucleus.

The preliminary results we showed in the previous section allowed us to establish some hypotheses regarding these questions, which will guide the direction of future works to be carried out using micropatterns of FN-bound BMP-2.

1. Matrix remodeling

Integrins are transmembrane receptors, part of a large family of cell surface receptors, and associated to cell adhesion to the extracellular matrix.

In the context of our study, the changes we observed in pattern organization after several days of culture (Figure 89) are a clear example of inside-out signaling occurring on our patterns of FN-bound BMP-2. Indeed, the FN in patterns of FN/BMP-2, initially homogeneously organized, was remodeled after 4 days of culture. The orientation of FN at this time mirrored that of the actin cytoskeleton, indicating an interaction between cytoskeletal tension inside the cell and ECM proteins outside.

These interactions between actin cytoskeleton and ECM proteins, and in particular FN, have previously been described. Indeed, studies have demonstrated a correlation between FN fibril distribution and the organization of submembranous bundles of actin and FA proteins (Hynes Fibronectin 1990). Furthermore, disrupting actin organization or alignment using

pharmacological agents like cytochalasin or glycated collagen was shown to similarly affect extracellular fibrillary FN (Figueroa, Kemeny, & Clyne, 2014). This fibrillogenesis was also shown to be affected by changes in myosin II activity, using pharmacological inhibition or enhancement with blebbistatin and calyculin A (Lemmon, Chen, & Romer, 2009). Finally, studies have demonstrated the impact of β1 activation in this intracellular tension-mediated ECM remodeling (Faurobert et al., 2013).

FN matrix assembly has been described in Figure 24 (Singh et al. 2010). Briefly, FN binds to α5β1 integrin, but not other RGD-dependent integrins (Huveneers, Truong, Fässler, Sonnenberg, & Danen, 2008), which leads to the clustering of integrins, thus promoting FN-FN interactions.

FN matrix assembly was shown to be linked to RhoA activity, and promoted by Lysophosphatidic acid (LPA) in the serum (Qinghong Zhang, Checovich, Peters, Albrecht, & Mosher, 1994). Indeed, LPA binding to receptors activates Rho (Anliker & Chun, 2004), which in turn leads to actin-myosin interactions and stress fiber formation, thus enhancing cell contractility (Hall, 2005). Inversely, the inhibition of RhoA or actin-myosin interaction prevents the assembly of the FN fibrillary matrix (Q Zhang, Magnusson, & Mosher, 1997; Zhong et al., 1998). These elements suggest that cytoskeletal tension is a key contributor to FN matrix assembly.

Supporting the bidirectional aspect of this integrin-mediated signaling, abnormal FN matrices also negatively affect cytoskeletal organization. Studies have shown that cells adhering to mutant FN matrices were affected in terms of cell growth, cell contractility, or barrier function in the case of an endothelial monolayer (Faurobert et al., 2013; Sechler & Schwarzbauer, 1998). Of note, these abnormal FN matrices can actually be more detrimental to the cells than having no FN at all. Other work on vascular smooth muscle cells demonstrated that cell growth was inhibited when FN matrix assembly was blocked with an anti-fibronectin monoclonal antibody (Mercurius & Morla, 1998).

Our results on patterns of FN/BMP-2 demonstrate the ability of cells spread on these patterns to remodel FN, and that the FN organization induced by this remodeling is parallel

to that of the actin cytoskeleton (Figure 90). We have also shown an implication of the RhoA/ROCK pathway in SMAD signaling on these patterns (Figure 82), as Wang et al. had shown using sBMP-2 in hMSCs on fibronectin-coated plates (Y.-K. Wang et al., 2012). These results suggest a strong role of RhoA/ROCK-mediated cytoskeletal tension in the generation of cellular forces responsible for the remodeling of the matrix.

From an inside-out perspective, it could therefore be interesting to further investigate the mechanisms guiding this remodeling, and how the differences we observed in cytoskeletal organization due to the presence and presentation mode of BMP-2 (Figure 73 and Figure 74) impact FN organization after several days of culture. Furthermore, inhibiting certain proteins in this pathway, like myosin II or LIMK, could provide some insight into how differences in FN matrix assembly on these patterns is impacted during the osteogenic differentiation of cells possessing this ability, such as C2C12 myoblasts or D1 MSCs.

Conversely, from an outside-in standpoint, and keeping in mind that our patterns remain stable and keep their overall shape after several days of culture, our technique could be a useful tool to investigate how FN matrix organization impacts undifferentiated cells. Differences in FN distribution induced by cell spreading can be interpreted as a way for the cell to record information, and leave a trace of its presence on the extracellular matrix. We have already seen that abnormal FN matrices can impact cell growth, but it is also possible that certain FN organizations can impact other cellular behaviors, like differentiation. Seeding cells on patterns with an already-established FN organization could possibly reveal such mechanisms of ECM- and integrin-mediated effects of the FN matrix on differentiation. This could be carried out through the lysis or trypinization of cells after several days of culture, followed by the seeding of new, undifferentiated cells on these modified patterns, and observing how cellular behaviors are altered by these matrices.

2. Focal adhesion recruitment and growth

The changes in the FN observed on our patterns led us to look more closely at the sites where the cell interacts with the ECM, namely focal adhesions.

Focal adhesions are dynamic structures linking integrins to the actin cytoskeleton (Benjamin Geiger et al., 2009). Indeed, F-actin in the cytoplasm has been shown to interact with the β subunit of integrin, with the intervention of numerous anchoring proteins (B Geiger et al., 2001; Zaidel-Bar et al., 2007).

a. Integrin involvement

Our preliminary results indicated that the β subunit of integrin was vital to cell spreading on patterns. More specifically, the importance of β1 in cell spreading on FN/BMP-2 patterns was demonstrated by the effect of silencing various integrin β subunits using a siRNA strategy (Figure 86). We observed a drastic effect of β1 silencing on C2C12 myoblast spreading, as cells stayed unspread, even after 4 hours of culture. Silencing of β3 or β5, however, did not appear to affect cell spreading in any discernable manner. In contrast, recently published work showed a β1-independent and β3-dependent spreading mechanism of C2C12 myoblasts on a soft matrix containing BMP-2 (Fourel et al., 2016). As had been the case with SMAD signaling, these discrepancies are likely due to differences in experimental setups, and we do not exclude a possible implication of β3 integrin in cell adhesion, spreading, or SMAD signaling for cells spread on our patterns. The involvement of β3 integrin is particularly relevant in the context of the stem cell niche and sensing of the underlying matrix, as it has been demonstrated that this integrin is involved in stiffness sensing (G. Jiang, Huang, Cai, Tanase, & Sheetz, 2006), a crucial aspect of stem cell fate determination (Engler et al., 2006).

To investigate this further, it would be interesting to see, using a siRNA strategy, how the silencing of various β subunits of integrin impacts the efficiency and kinetics of cell adhesion on different patterns:

- Live imaging of these cells during the first hour after seeding could reveal certain mechanisms at play, and early signaling mechanisms at the cell membrane.
- Using patterns of FN/BMP-2 and FN with or without sBMP-2 could also highlight how BMP-2 presentation mode leads to the recruitment of different integrins.

- Looking at SMAD signaling in these cells could reveal which integrins are involved in this signaling pathway, and if the mechanisms of adhesion on the patterns truly link to downstream signaling events like osteogenic differentiation.
- Finally, silencing various combinations of integrins could be a useful way to investigate potential compensation mechanisms between integrins, where the silencing of one would lead to the overexpression of another.

While these questions remain unanswered, we obtained some preliminary observations regarding the issue of adhesion kinetics and early signaling events. Indeed, we observed a distinctly more rapid cell adhesion and spreading on patterns of FN-bound BMP-2, when compared with cells spread on FN alone, regardless of the presence of sBMP-2.

These results are shown in Figure 85, and confirm a crucial role of BMP-2 presentation mode in early mechanisms of adhesion and spreading. Having already observed a role of β1 integrin in this cellular behavior, and knowing that proteins involved in focal adhesion formation and growth play a crucial role in the ability of a cell to adhere and spread (Bays & DeMali, 2017; Carisey & Ballestrem, 2011; DeMali, 2004; Ezzell, 1997; Van der Gaag et al., 2002; West et al., 2001), we decided to investigate further the role of FA proteins, and in particular vinculin.

b. Vinculin

As described in the introduction, vinculin is a protein involved in focal adhesions, and part of the chain linking integrins to the actin cytoskeleton, as shown in Figure 93 (Turner, 2000).

Vinculin's interaction with talin (Burridge & Mangeat, 1984; Cohen et al., 2005), the increased recruitment of vinculin and talin molecules to focal adhesions after binding, and the link between talin and the β1 subunit of integrin (Horwitz et al., 1986; Johnson & Craig, 1994) demonstrate a clear link between vinculin and β1 integrin.

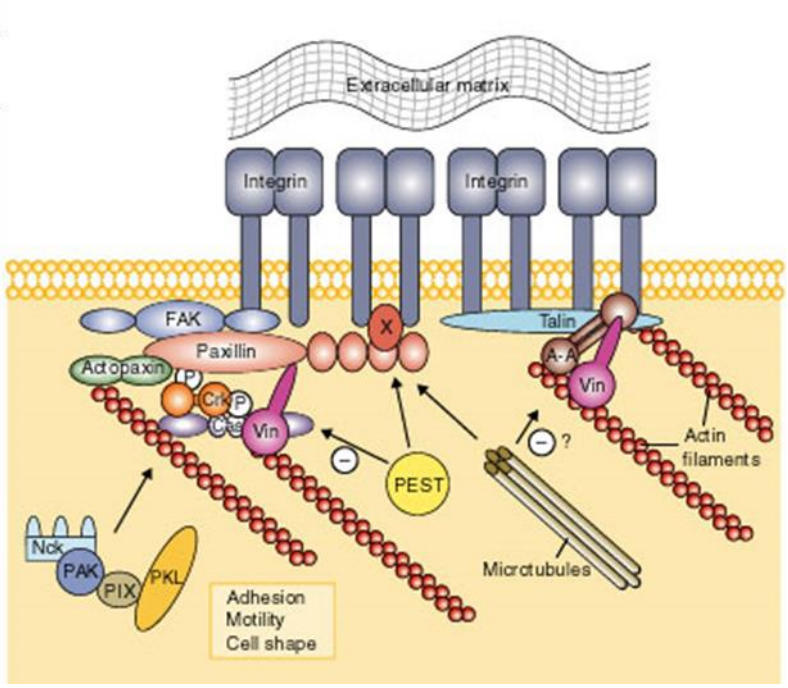


Figure 93: ***Integrin signalling and interactions with the actin cytoskeleton*** *Integrins are linked to the actin cytoskeleton via talin and α-actinin (A-A). (The regulation and protein composition of focal adhesions is more complex than depicted.) Adapted from Turner 2000.*

We have already seen that the β1 subunit of integrin is important to cell spreading on our patterns. Furthermore, our results show a drastic change in focal adhesion organization depending on BMP-2 presentation mode. When presenting BMP-2 in a FN-bound manner, vinculin appeared much more homogeneously distributed under the cell, whereas on patterns of FN vinculin was mainly present at the edges of the cell, regardless of the presence or not of sBMP-2 (Figure 73).

This distribution of vinculin, combined with the well-documented importance of vinculin in cell spreading (Carisey & Ballestrem, 2011; DeMali, 2004; Ezzell, 1997), suggest that BMP-2 presentation mode impacts the previously-described adhesion and spreading kinetics (Figure 85) through changes in focal adhesion organization. It therefore appears that certain BMP-2-induced signaling pathways only occur when BMP-2 is presented bound to the ECM.

BMP-2 signaling occurs via the intervention of a BMP-receptor complex, which is the functional unit required for downstream signaling events. This complex is comprised of two transmembrane serine/threonine kinases, named BMPR type I (BMPR-I) and type II (BMPR-II) (Hoodless et al., 1996; Kawabata, Chytil, & Moses, 1995; F. Liu, Ventura, Doody, &

Massagué, 1995; Nohno et al., 1995; Rosenzweig et al., 1995). Of note, activated BMPR-I are responsible for the phosphorylation of SMAD1/5/8, which then forms a complex with SMAD4, and is translocated into the nucleus during the early stages of osteogenic differentiation (Attisano & Wrana, 2000; Y. Chen, 2000; Nohe et al., 2002).

As described above, we noticed a marked increase in nuclear p-SMAD1/5/8 in cells spread on our patterns, suggesting an activation of BMPR-I. Interestingly, studies on astrocytes using co-immunoprecipitation have demonstrated a direct interaction between β1 integrin and bone morphogenetic protein receptors (BMPR), and more specifically subunits BMPR1a and BMPR1b (North et al., 2015). While we did not directly image BMP receptors in the context of this study, it would be interesting to pursue this avenue of research, especially considering recent works which demonstrated crosstalk between BMPR-Ia and β3 integrin (Fourel et al., 2016). Some of these potential perspectives are developed below.

As a reminder, we had observed that the silencing of β1 integrin using a siRNA strategy had a drastic effect on cells spread on patterns of FN-bound BMP-2 (Figure 86). Cells were unable to spread, and remained small. These data suggest that the vinculin organization observed on our patterns of FN/BMP-2 is likely the result of a crosstalk between β1 integrin and BMPRs. Although we have not directly quantified the amount of vinculin for each printing condition, this crosstalk could induce β1 integrin activation, and thus increased vinculin recruitment (Byron et al., 2015). This would lead to an increased vinculin-talin interaction, and thus a stronger FA growth than in patterns of FN alone (Humphries et al., 2007). At the very least this crosstalk would be responsible for the more homogeneous distribution of vinculin in these cells. Of note, presenting BMP-2 in solution would not rescue this phenotype, as this presentation mode does not permit β1/BMPR crosstalk at adhesion sites.

This hypothesis needs to be confirmed, first by precisely quantifying the adhesion kinetics depending on the presence or not of BMP-2, and more importantly depending on its presentation mode (FN-bound or in solution). This can easily be done on patterned substrates like microcontact printed (PLL/HA) films, as spreading is limited by pattern size, and it is therefore easy to assess when maximum spreading of the cell is achieved.

To investigate this supposed crosstalk, we could use immunofluorescent staining of integrins and BMPRs to follow colocalization, and fluorescence resonance energy transfer (FRET) to reveal if a direct interaction between these two cell membrane receptors is occurring. Furthermore, the impact of BMPRs in this observed phenomenon could be investigated by silencing BMPRs using a siRNA strategy, and investigating how this silencing affects adhesion and spreading kinetics on the one hand, and vinculin organization on the other hand. We suspect that silencing BMPR-I would suppress the increased spreading speed observed for cells on patterns of FN-bound BMP-2.

As mentioned above, vinculin binds to actin (Golji & Mofrad, 2013). It also plays an important role in mechanically coupling integrins to the cytoskeleton (Ezzell, 1997), and vinculin levels in FAs have been shown to correlate linearly with traction forces (Balaban et al., 2001). Considering the important differences in vinculin distribution we had observed on our various patterns, we decided to investigate further how these differences affected the organization of the actin cytoskeleton, and how the presence of BMP-2 and its presentation mode impacts the forces applied by cells to their underlying substrate.

3. Cytoskeletal organization and mechanical coupling

a. Traction forces on the underlying substrate

The elements discussed thus far indicate a hypothetical mechanism underlying cell adhesion, spreading, and signaling pathways, based on potential integrin/BMPR crosstalk. The vinculin distribution and actin organization on our patterns also suggest a role of cytoskeletal reorganization and mechanotransduction in these cellular behaviors.

Indeed, crosstalk between β1 integrin and BMPR-I, and the subsequent increased FA growth – mediated by vinculin recruitment – could explain the faster adhesion kinetics of C2C12 myoblasts on FN/BMP-2 patterns, the inability of siβ1-treated cells to spread, as well as the differences in vinculin distribution and actin organization surrounding the nucleus. This hypothesis also provides an explanation as to why sBMP-2 does not induce similar behaviors

in cells spread on patterns of FN alone, due to the distance between activated BMPRs on the dorsal side of the cell and FAs on its basal side.

Using traction force microscopy, we measured the contractile energy in cells spread on FN/BMP-2, or FN alone with or without sBMP-2 (Figure 91), and observed a similar trend to that of nuclear p-SMAD1/5/8 in these cells (Figure 77). These data are summarized in Figure 94. Indeed, the presence of BMP-2, regardless of its presentation mode, seemed to increase the traction forces exerted by the cell on its underlying matrix. As had been the case for SMAD signaling, this increase was particularly important in the presence of sBMP-2.

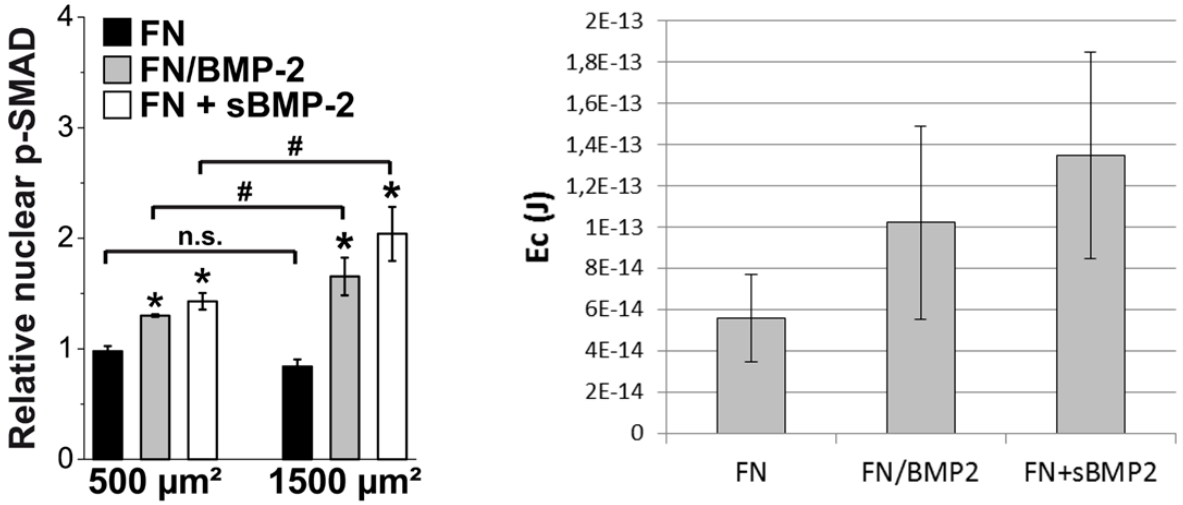


Figure 94: ***Possible link between SMAD signaling and traction forces****. Left: Quantification of the relative nuclear p-SMAD1/5/8 (n > 100 cells) in function of the size and composition of the micropatterns. * p < 0.01 versus negative control (FN patterns without BMP-2 in solution); # p< 0.01 between small and large micropatterns. n.s. stands for non-significant (i.e. p > 0.01). Right: Contractile energy (in J) of cells spread on homogeneously patterned gels of polyacrylamide (E=14kPa), highlighting the effect of the presence of BMP-2 and its presentation mode on cellular forces.*

The mechanism underlying a potential link between traction forces and SMAD signaling still remains unclear. Based on previous studies showing that vinculin levels within FAs correlate in a linear manner with traction forces (Balaban et al., 2001), our initial hypothesis was that traction forces generated by integrins at the adhesion sites are mechanically coupled to the cytoskeleton through vinculin (Ezzell, 1997), and that these forces, transmitted to the nucleus through the LINC complex, lead to increased nuclear translocation of p-SMAD1/5/8, as shown in our results (Figure 77) as well as other studies (Y.-K. Wang et al., 2012).

While this may be true when BMP-2 is presented in a FN-bound manner, on which vinculin is expressed over the entire surface of the cell, it does not explain the results we observed when we seeded cells on patterns of FN alone with BMP-2 in solution. Indeed, despite expressing vinculin only on their edges, these cells exerted higher traction forces on the underlying substrate (Figure 91) and more nuclear translocation of p-SMAD1/5/8 than cells on FN/BMP-2 (Figure 77).

To explain these observations, we established three hypotheses:

- Recently published work indicates that, in experimental conditions similar to ours, BMP-2 endocytosis is higher when presented In solution (Gilde et al., 2016). In a pathway quite distinct from the one described above, this presence of internalized BMP-2 in the cytoplasm could potentially induce inside-out signaling to integrins. Indeed, the stimulation of the cytoplasmic domain of integrins at the cell membrane can lead to a change in its conformation, which is transmitted to its extracellular domain (Ginsberg, Du, & Plow, 1992; Hynes, 2002; J. Qin et al., 2004; Schoenwaelder & Burridge, 1999). This cytoplasmic signaling to integrins could explain why cells spread on patterns of FN in the presence of sBMP-2 exert higher forces on their underlying substrate than those spread on patterns of FN/BMP-2, and this despite vinculin expression being only at the edges of these cells. This explanation is conceivable, and to some extent supported by studies carried out on hMSCs, which showed an increase in traction forces in presence of sBMP-2 (Y.-K. Wang et al., 2012).

- Another explanation for this observation could be due to a bias in measurement in our TFM results. The contractile energy of cells is calculated based on the in-plane bead displacement in images taken before and after cell lysis or trypsinization (Dembo & Wang, 1999). While TFM is a standard method to study forces for both individual cells and tissue-like cell sheets, it functions based on the projection of displacement vectors on a 2D plane. Therefore, it neglects the out-of-plane or normal component of the traction forces exerted by the cell on its underlying substrate. Considering the differences in terms of vinculin we observed between our different conditions, and more specifically the homogeneous distribution of vinculin with patterns of FN/BMP-2, we may be underestimating the forces applied by the cell on

these patterns, in particular in comparison with cells on FN patterns in presence of sBMP-2.

Although we are currently unable to provide evidence supporting that this is indeed the case in the work we present here, studies focusing on this issue have been carried out, thus indicating its technical and biological relevance (del Álamo et al., 2013). The development of new tools, like three-dimensional force microscopy (Hur, Zhao, Li, Botvinick, & Chien, 2009; Legant et al., 2010, 2013; Steinwachs et al., 2016) could be useful for a more accurate comparison of forces between these different conditions.

- Finally, these perceived discrepancies in terms of traction forces could simply be due to the differences in actin organization between our different conditions. Indeed, on patterns of FN, the presence of sBMP-2 induced an increase in the cytoskeletal tension of C2C12 myoblasts, as had previously been demonstrated by Wang et al. using hMSCs (Y.-K. Wang et al., 2012). An increase was also observed in the presence of FN-bound BMP-2, albeit lower than with sBMP-2. The activation of the RhoA signaling pathway explains why the presence of BMP-2, whether in solution or matrix-bound, induces an increase in traction forces.

  When BMP-2 was presented in solution, vinculin was only present at the edges of cells, and this resulted in a more highly polarized actin cytoskeleton.

  When BMP-2 was presented in a FN-bound manner, however, vinculin was presented homogeneously under the cell, resulting in less uniformly distributed stress fibers.

  This loss of polarization of the actin cytoskeleton on FN containing BMP-2 is consistent with other studies investigating the effect of polarization on force patterns (Mandal et al., 2014), and strikes us as the most likely explanation of the changes in contractile energy observed in our cells.

In any case, the results which led us to these three hypotheses are extremely preliminary, and are therefore to be interpreted with caution. Furthermore, the underlying mechanisms need to be elucidated, and supported by clear evidence before drawing any conclusions.

One way of investigating this phenomenon would be to use square hollow patterns, like those shown in Figure 80. On these patterns, cells would express vinculin only at the edges of the cell, and there would no longer be any focal adhesions in the center of the cell. This would allow us to decouple BMP-2 presentation mode and vinculin localization. We suspect that in these conditions cells on patterns of FN/BMP-2 would present similar traction forces as those on patterns of FN alone with sBMP-2, and that both these forces would be higher than in the absence of BMP-2. If this is indeed the case it would indicate that focal adhesion sites – observed via vinculin localization – and the resulting actin organization are responsible for the traction forces exerted by cells on their underlying substrate.

Regardless of the mechanisms at play, these differences in cellular tension depending on BMP-2 presentation mode seem to correlate with levels of nuclear p-SMAD1/5/8. We therefore suspect that a link exists between traction forces exerted by the cell, and the nuclear translocation of p-SMAD1/5/8. This mechanical coupling is likely transmitted from the cell membrane to the nucleus, through integrins, vinculin, and the actin cytoskeleton.

b. Mechanotransduction to the nucleus

When imaging the cytoskeleton, our results showed a distinct difference in actin organization between cells spread on patterns of FN/BMP-2 and patterns of FN, regardless of the presence or not of sBMP-2. Indeed, the differences we had initially observed on average actin images (Figure 73) were later confirmed using z-profiles of actin and nuclear fluorescence, which highlighted the presence of increased actin surrounding the nucleus when BMP-2 was presented in a matrix-bound manner (Figure 87).

These results are coherent with the differences we had already observed in terms of vinculin organization (Figure 73), and keeping in mind that vinculin presents binding sites to F-actin (Golji & Mofrad, 2013). This organization surrounding the nucleus does however open an interesting line of questioning regarding the mechanical coupling of the membrane to the nucleus. Indeed, and as described in the introduction, we know that forces are transmitted from the cell membrane to the nucleus, propagated via integrins and the actin cytoskeleton, as was shown in Figure 26 (Maniotis et al., 1997; N. Wang et al., 2009).

Furthermore, we know that the LINC complex connects the nucleus to the cytoplasm, and is involved in mechanotransduction to the nucleus (Lammerding et al., 2004; Rowat et al., 2006), and that lamin A and LEM domain proteins like SANE modulate signals from the BMP signaling pathways (Raju et al., 2003; Tsukune et al., 2017).

Of note, the nuclear translocation of the SMAD4-p-SMAD1/5/8 complex is carried out through the intervention of specific nucleoporins, allowing its transport through the nuclear pore complex (NPC) (X. Chen & Xu, 2010). If the mechanical signals regulating SMAD signaling are transmitted to the nucleus through the LINC complex, a possible explanation for increased nuclear p-SMAD1/5/8 on larger patters is that this mechanotransduction translates to changes in nucleoporin expression or function, resulting in increased nuclear translocation. A possible way of investigating this hypothesis would be to disrupt elements of the LINC complex, and to follow SMAD signaling in cells spread on our patterns. A decrease in nuclear p-SMAD1/5/8 in these cells would not necessarily confirm the implication of nucleoporin as a downstream player in the cellular response to cytoskeletal tension, but would however indicate a fundamental role of mechanotransduction through the LINC complex in these cells.

In any case, taken together these results demonstrate the importance of mechanotransduction in the outside-in communication which travels from focal adhesions – and more specifically integrins – to the nucleus, via the actin cytoskeleton. This means of communication allows the regulation of signaling pathways and complex cellular behaviors such as osteogenic differentiation; this puts into perspective the biological relevance of our preliminary results in terms of integrin involvement, adhesion kinetics and mechanics, and actin orientation.

CONCLUSIONS AND PERSPECTIVES

I. Conclusions

In the works described above, we attempted to recreate a simplified niche model to study, at the single cell level, the factors influencing certain cell behaviors like adhesion, cytoskeletal organization and differentiation. To accomplish this, we used biomimetic films, obtained through the layer-by-layer deposition of PLL and HA, as well as micropatterns of BMP-2 within or without a FN matrix. Using a combination of these two techniques, we investigated the ability to generate highly reproducible patterns, but also the adhesion, spreading and cytoskeletal organization of cells on these patterns. Furthermore, we studied the ability of micropatterned BMP-2 to induce osteoblastic differentiation in several cell types (C2C12 myoblasts, D1 MSCs and human myoblasts), as well as the signaling pathways involved in this differentiation. We also looked at potential links between cell forces and BMP-2 presentation mode, adhesion kinetics, integrin involvement in cell spreading on micropatterns, and bidirectional signaling at the cell membrane through matrix remodeling.

1. Pattern characterization

By associating microcontact printing with either LbL films or polyacrylamide hydrogels, we were able, for the first time, to generate cellular- and subcellular-sized micropatterns of unmodified BMP-2 within or without FN on soft biopolymeric films and hydrogels. We demonstrated the ability to routinely produce arrays of BMP-2 and FN-bound BMP-2 micropatterns with a micrometric resolution (~ 3 µm).

2. Cell adhesion and spreading

Using these BMP-2 micropatterns, we examined the effect of pattern size on cell adhesion, spreading, and cytoskeleton organization, with single-cell precision.

These works showed that cells selectively adhered to the micropatterns, and spread fully when BMP-2 was trapped in a FN matrix. Cell adhesion was possible when BMP-2 was printed alone, although we observed limitations in terms of spreading, particularly on larger patterns.

By printing FN-bound BMP-2, we were able to generate cell adhesive islands on an otherwise non-cell adhesive surface, and these islands could be used to control cell spreading at a single cell level.

Preliminary observations of adhesion and spreading kinetics showed that cell spreading occurred faster on patterns of FN-bound BMP-2, and that this behavior was not exhibited by cells spread on FN alone in the presence of sBMP-2. Based on these results, and the deleterious effect on cell spreading we observed when silencing β1 integrin expression, we hypothesized that cell adhesion on these patterns was mediated by β1 integrin, and potentiated by BMPR recruitment and crosstalk with integrins when presenting BMP-2 in a matrix-bound manner.

3. Cytoskeletal organization

Using patterns of different sizes, we were able to show an effect of cell spreading on cytoskeletal organization, with the presence of diagonal stress fibers reinforcing the cytoskeleton on larger patterns.

Furthermore, this cytoskeletal organization seemed to be impacted by the presentation mode of BMP-2, as evidenced by the strong presence of actin surrounding the nucleus when presenting BMP-2 in a matrix-bound manner, which was not the case when presenting BMP-2 in solution.

4. Osteoblastic differentiation

These works demonstrated that the spatially patterned, FN-bound presentation of BMP-2 and the resulting cell spreading regulate the phosphorylation and translocation to the nucleus of SMAD1/5/8 in myoblasts, through the activation of LIM kinase (LIMK) via the Rho-associated kinase (ROCK) pathway. They also showed that this signaling pathways was mediated by LIMK2 rather than LIMK1.

Similar observations in terms of nuclear translocation of p-SMAD1/5/8 were also observed in D1 murine MSCs.

5. Cellular forces

Preliminary results suggest that mechanotransduction plays a role in regulating BMP-2-induced SMAD signaling. This was evidenced by higher contractile forces being exerted in the presence of BMP-2, and following the same trend that we had observed for the nuclear translocation of p-SMAD1/5/8.

These forces, which are transmitted to the nucleus through the actin cytoskeleton and LINC complex, likely regulate the BMP-2-induced osteoblastic differentiation of these cells.

6. Matrix remodeling

We were able to observe, in preliminary experiments, evidence of integrin-mediated inside-out signaling, through the remodeling of the pattern after several days of culture. While the pattern maintained its overall shape, the FN, which was initially homogeneously distributed, was reorganized into fibrils that followed similar orientations as the actin cytoskeleton. This reorganization of the matrix could therefore be induced by contractile forces exerted by the cell, and is likely involved in the regulation of signaling pathways and cell behaviors like differentiation.

Taken together, these results show how we were able to use patterning of soft biomimetic substrates to control the microenvironment of the cell, and to use this to control cell behavior, in particular differentiation. These results open an exciting avenue for studying the mechanisms of integrin/BMPR crosstalk and mechanotransduction involved when BMP-2 is presented by the ECM. Thus, microcontact printing of BMP-2 on soft biopolymeric films could become a useful tool for studying the impact of matrix-bound BMP-2, from its interaction with the ECM to cell adhesion and differentiation, in reproducible and standardized conditions. Most importantly, our approach could be adapted to other combinations of ECM proteins and GFs, depending on their respective affinity (Mikael M

Martino et al., 2014), providing valuable opportunities to recreate tissue-specific microenvironments in vitro and analyze stem cell renewal and differentiation in well-defined conditions.

II. Future works

Recreating and understanding the various parameters that affect cells in their matrix is crucial in order to fully understand how this particular microenvironment regulates cell differentiation. Conversely, it can provide valuable information to decipher the mechanisms by which this microenvironment is generated and maintained by the cells.

1. Technical optimization

a. Printing

From a technical standpoint, one of the limitations in generating micropatterns using microcontact printing is reproducibility and user-dependency, as well as the time-consuming aspect of manually printing using PDMS stamps. This can be quite prohibitive when attempting to screen different ECM protein/growth factor combinations, or even different patterns sizes and shapes. Automatization, using commercially available or custom-made systems can be an efficient way to facilitate the printing process and use these patterns for high-throughput screening.

Regarding commercially available systems, a company called Innopsys has developed a device named Innostamp (Figure 95) which allows microcontact printing in a rapid, reproducible, and controlled manner, and with limited user interaction, thus rendering the entire process far less time-consuming.

*Figure 95: **Innostamp 40**. From the Innopsys website.*

Indeed, Innopsys kindly allowed us to carry out experiments using the Innostamp to generate patterned substrates. While the automated printing process in itself was not necessarily faster than printing manually, one of the main advantages of this technique is that it only requires preparation work from the user, who can then load multiple samples into the device, and collect the micropatterned substrates once the printing cycle is complete. Furthermore, by allowing the user to control the temperature, humidity, and even force applied during printing, the process can be optimized to best suit the proteins and substrates used, and the same parameters can then be used for all subsequent experiments.

Facilitating the printing process in this manner could make microcontact printing readily available for biologists for example, who may not wish to focus on the materials or technical aspects of generating micropatterns, but rather on the biological questions that can be answered using patterned substrates.

b. Image acquisition and analysis

Another important aspect of using micropatterns is image acquisition and data analysis. Indeed, micropatterning generates arrays of hundreds of patterns, and because cell sizes and shapes on these patterns are normalized, it is possible, using a single substrate, to acquire many images of single cells spread on patterns. While this allows a strong statistical

robustness of results, it is also highly time-consuming. A similar issue is encountered when extracting and analyzing data from the acquired images. Automatization of these processes is a vital aspect in order to get the most out of patterned substrates. Because of the regularity of patterns in the array, it is possible to configure the acquisition software to automatically scan the entire matrix of patterns on the substrate, without the need for user intervention. Likewise, it is possible to develop macros and programs on software like ImageJ and Matlab, which allow the automated analysis of the images.

By automatizing the repetitive aspects of the printing, acquisition and analysis steps, it is thus possible to use the micropatterning tool in the most efficient manner possible.

2. Mechanotransduction

One of the crucial aspects in the regulation of cell behavior is the transmission of mechanical cues from the ECM to the cell, and specifically how these forces are sensed by the cell at the membrane and transmitted to the nucleus via the cytoskeleton. Future works on these patterns could investigate the mechanisms by which these forces are transmitted, but also how BMP-2 presentation mode affects cell forces, possibly through integrin/BMPR crosstalk.
A first step in investigating these interactions could simply be to carry out immunofluorescent staining and confocal microscopy imaging of BMPRs and β1 integrins, to investigate how the presentation mode of BMP-2 affects their distribution. Based on all the elements described above, the hypothesis would be that when presenting BMP-2 in a matrix-bound manner, there would be a strong co-localization between BMPRs and β1 integrins, suggesting possible interactions between these two transmembrane proteins.

However, co-localization is not direct evidence of interaction, so this interaction would need to be confirmed, possibly using Fluorescent Resonance Energy Transfer (FRET). This technique, which uses fluorescent probes attached to macromolecules like proteins, only functions when donor and acceptor probes are in very close contact, i.e. under 100 Å. Because of this, it reflects protein interactions (dos Remedios, 1995; Kenworthy, 2001; Remedios, 2015; Stryer, 1978). It can also be carried out in live imaging, thus allowing the tracking of changes in protein interactions over time. These interactions could then be

correlated with local changes in force distribution using TFM, to investigate how BMPR/integrin interactions affect the forces exerted by the cell on its substrate, at a highly localized level.

Since the transmission of mechanical forces is both outside-in and inside-out, studying how these forces relate to the remodeling of the matrix, and conversely how a remodeled matrix can affect cell behavior would allow us to further understand how the cell communicates mechanically with its microenvironment. Studies investigating the effect of matrix remodeling on cell behavior have shown very promising results regarding the effect of remodeled matrices on invasion in cancer (Gaggioli et al., 2007) or cerebral cavernous malformations (Faurobert et al., 2013). These studies highlight how matrix organization is crucial in regulating cell behaviors.

In the context of our study, seeding cells on patterns already remodeled by differentiated cells and investigating the effect of matrix organization on the guidance of stem cell fate could provide valuable insight in the role of the ECM in differentiation. Furthermore, differentiating the FN deposited during the printing process and FN deposited by the cell could demonstrate how the cell adapts to the presence of a pre-existing matrix in order to regulate its own production of ECM proteins.

Similarly, using cells which secrete BMP-2 (such as MSCs), we could follow how the cell itself deposits this growth factor on the FN patterns, and in particular how the FN organization on the pattern (homogeneously distributed versus remodeled into fibrils) affects BMP-2 localization. This could easily be investigated using immunofluorescent staining and confocal microscopy on fixed cells, by staining for BMP-2. On patterns of FN-bound BMP-2, this could further be investigated by using fluorescently labeled BMP-2 for the printing process, and a different fluorophore during immunofluorescent staining, thus showing potential differences in BMP-2 localization between the printed and the secreted BMP-2, and thus an influence of ECM organization.

3. Other ECM protein/growth factor combinations

As mentioned above, we were able to print BMP-2 bound with FN due to the specific and high affinity between this growth factor and this ECM protein. Other combinations of growth factors and ECM proteins could however be used to study other stem cell niches, or even decouple the contribution of each protein on the synergistic effect observed.

a. Modulating the ECM protein to study osteoblastic differentiation

In the context of osteoblastic differentiation and the MSC niche, similar experiments as the ones described with FN could be carried out using ECM protein Tenascin-C.

Tenascin-C is a glycoprotein of the ECM which is expressed in many different physiological and pathological tissues, in different stages of development and throughout the body (brain, skin, lung, bone, cartilage). A detailed review of Tenascin-C, its localization, structure, cellular interactions and expression has been carried out elsewhere (Midwood, Chiquet, Tucker, & Orend, 2016).

More specifically, Tenascin-C has been shown to be present in the endosteum (the connective tissue which is present at the inner surface of bone tissue) and the periosteum (the membrane covering the outer surface of bone tissue), but is not present in mineralized bone tissue (Mackie, Thesleff, & Chiquet-Ehrismann, 1987; Thesleff, Kantomaa, Mackie, & Chiquet-Ehrismann, 1988). Furthermore, it has been shown that Tenascin-C is synthesized by osteoblasts (Mackie & Tucker, 1992), and that this ECM protein plays a role in osteoblastic differentiation by maintaining ALP activity and collagen synthesis (Mackie & Ramsey, 1996). Further roles of Tenascin in the regulation of osteoblast behavior and the osteogenic stem cell niche have been reviewed elsewhere (Chatakun et al., 2014; Chiquet-Ehrismann, Orend, Chiquet, Tucker, & Midwood, 2014).

This role of Tenascin-C in the regulation of osteoblastic differentiation is an interesting question when recreating the stem cell niche, as it has been shown that Tenascin-C has an affinity for BMP-2 (Mikael M Martino et al., 2014) and, as mentioned above, BMP-2 has been

shown to play a role in all stages of osteoblastic differentiation (H. Cheng et al., 2003). Furthermore, BMP-2 treatment has been shown to increase Tenascin-C expression in pre-osteoblastic cells (Morgan, Wong, Yellowley, & Genetos, 2011). These showings suggest that we could generate micropatterns of Tenascin-C with or without BMP-2, in order to decouple the effect of this ECM protein and the growth factor on cell behavior, and in particular at later stages of osteoblastic differentiation.

Of note, the fact that Tenascin-C is both induced by and induces osteoblastic differentiation makes it particularly interesting to study bidirectional signaling between the cell and the ECM during processes like stem cell differentiation.

Furthermore, the Tenascin-C/BMP-2 combination could be compared to other ECM protein/BMP-2 combinations, such as Fibronectin/BMP-2 (as described in the works above), or Laminin/BMP-2.

Indeed, certain laminins have been shown to promote osteogenic differentiation (Mittag et al., 2012), and preliminary trials carried out in the context of this thesis have shown that it is possible to pattern laminin on a biomimetic film using microcontact printing, and that laminin was able to retain BMP-2 over time.

Indeed, and as shown in Figure 96, it is possible to obtain well-defined patterns and a good transfer of BMP-2 using this combination of proteins.

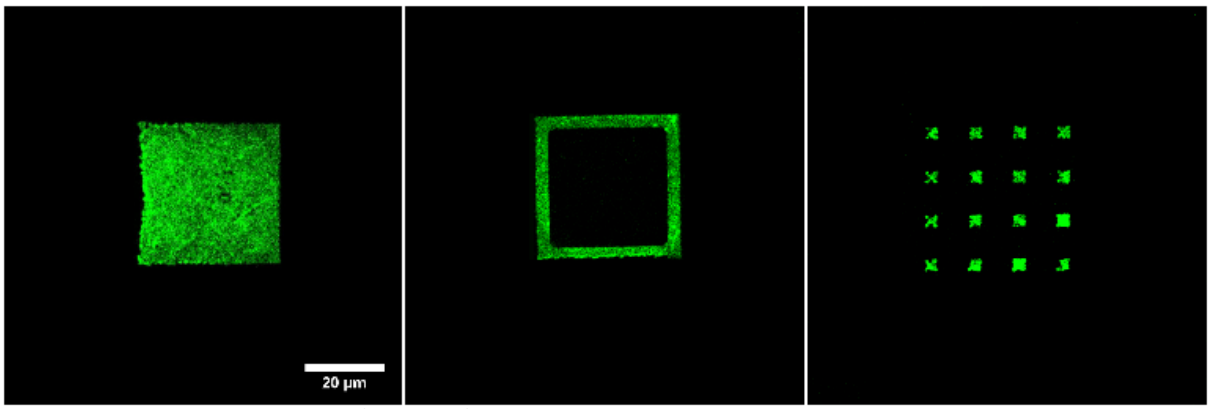


*Figure 96: **Patterns of LN/BMP-2^{CF}**. BMP-2^{CF} is in green.*

Furthermore, as with FN, BMP-2 is immobilized when printed in a LN-bound manner (Figure 96).

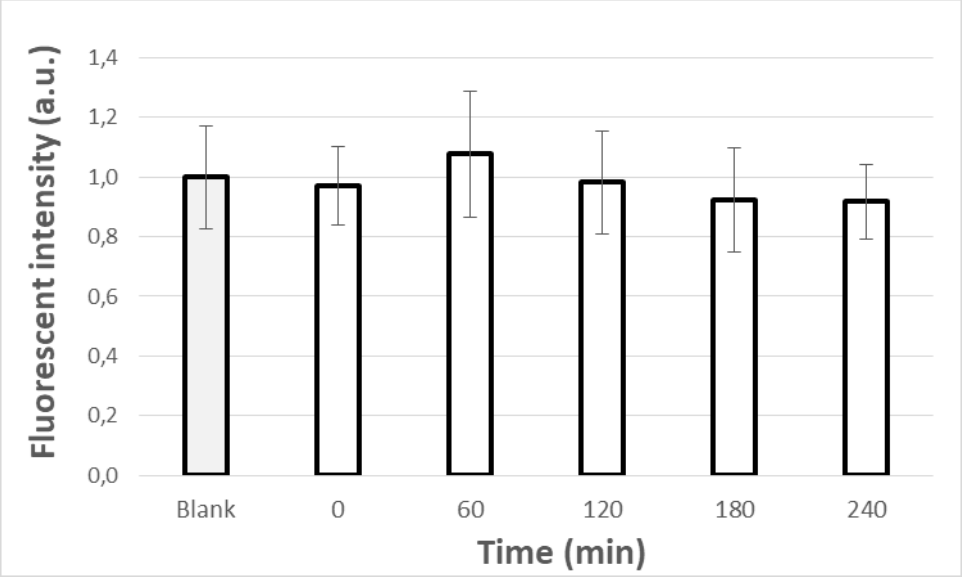


Figure 97: ***Release of BMP-2^{Cf} from patterns of LN/BMP-2 for 4 hours at 37 °C****. Excitation: 492 nm; Emission: 517 nm.*

Indeed, no significant release of BMP-2 was observed from patterns of LN/BMP-2 over the course of 4 hours at 37 °C. Cells spread on these patterns would display a very different integrin repertoire than cells spread on patterns of FN/BMP-2. Using these patterns could thus be a useful way to dissect how ECM proteins and therefore integrins influence and potentiate BMP-2-induced signaling.

b. Tenascin-C, TGF-β1 and BMP-2 in the context of stem cell fate differentiation

Tenascin-C can also be used in recreating the MSC niche microenvironment through its affinity with growth factor TGF-β1 (Mikael M Martino et al., 2014). This affinity can be used to investigate stem cell fate determination in the context of osteogenesis versus chondrogenesis. This topic is of particular interest due to the fact that in vivo studies have suggested complex mechanisms in which cells from the periosteum, endosteum and bone marrow participate, through a combination of intrinsic and environmental factors, in both osteogenesis and chondrogenesis during bone repair (Colnot, 2009).

As mentioned above, Tenascin-C is present in unmineralized bone tissue (Mackie et al., 1987; Thesleff et al., 1988), but it is also present in articular cartilage (Hasegawa, Yoshida, & Sudo, 2017). Furthermore, Tenascin has been shown to be associated with both chondrogenic and osteogenic differentiation in vivo (Mackie et al., 1987), and we know that several cell types such as MSCs or periosteal cells display both chondrogenic and osteogenic potential in vitro and in vivo (Kawanami, Matsushita, Chan, & Murakami, 2009; Reagan & Rosen, 2015).

Tenascin induces osteogenic and chondrogenic differentiation in undifferenciated stem cells, and is also expressed by committed cells, and notably osteoblasts (Mackie & Tucker, 1992), and chondrocytes (Ghert, Qi, Erickson, Block, & Scully, 2002; Savarese, Erickson, & Scully, 1996).

TGF-β1 is a growth factor which, like BMP-2, is part of the TGF-β superfamily. As demonstrated in Figure 98 (Kasagi & Chen, 2013), TGF-β1 plays many different roles in the bone. Interestingly, it has been demonstrated to either stimulate or inhibit bone formation, and to affect in very different manners osteoblast markers in vitro (Breen et al., 1994). Furthermore, this growth factor has also been shown to stimulate MSC proliferation and their differentiation into chondrocytes (Eaves et al., 1991).

The apparently contradictory and multiple effects of TGF- β1 in vitro and in vivo (Karsenty, 1999; Kasagi & Chen, 2013) are likely due to the complexity of the various roles played by this growth factor. Our hypothesis is that the local microenvironment, and thus the stem cell niche, will guide the cell in determining its fate in presence of TGF-β1.

A way to decouple the opposite effects of TGF-β1 and BMP-2 described in the literature (Spinella-Jaegle et al., 2001) could be to use patterns as a tool to decipher the effect of both growth factors by combining them at different concentrations. This initial screening, with normalized cell shape and spreading, would therefore study the effect of each growth factor and their combination independently of other cues such as changes in cell geometry and cytoskeletal tension. Further down the line, adding changes in cell geometry and tension as well as matrix stiffness may actual provide additional information regarding how MSCs

determine their fate between these cells which function in tissues with very different mechanical properties, i.e. cartilage and bone.

By using combinations of ECM proteins and combinations of growth factors to generate the patterns, we may be able to further decipher the separate and synergistic effects of these different components of the stem cell niche.

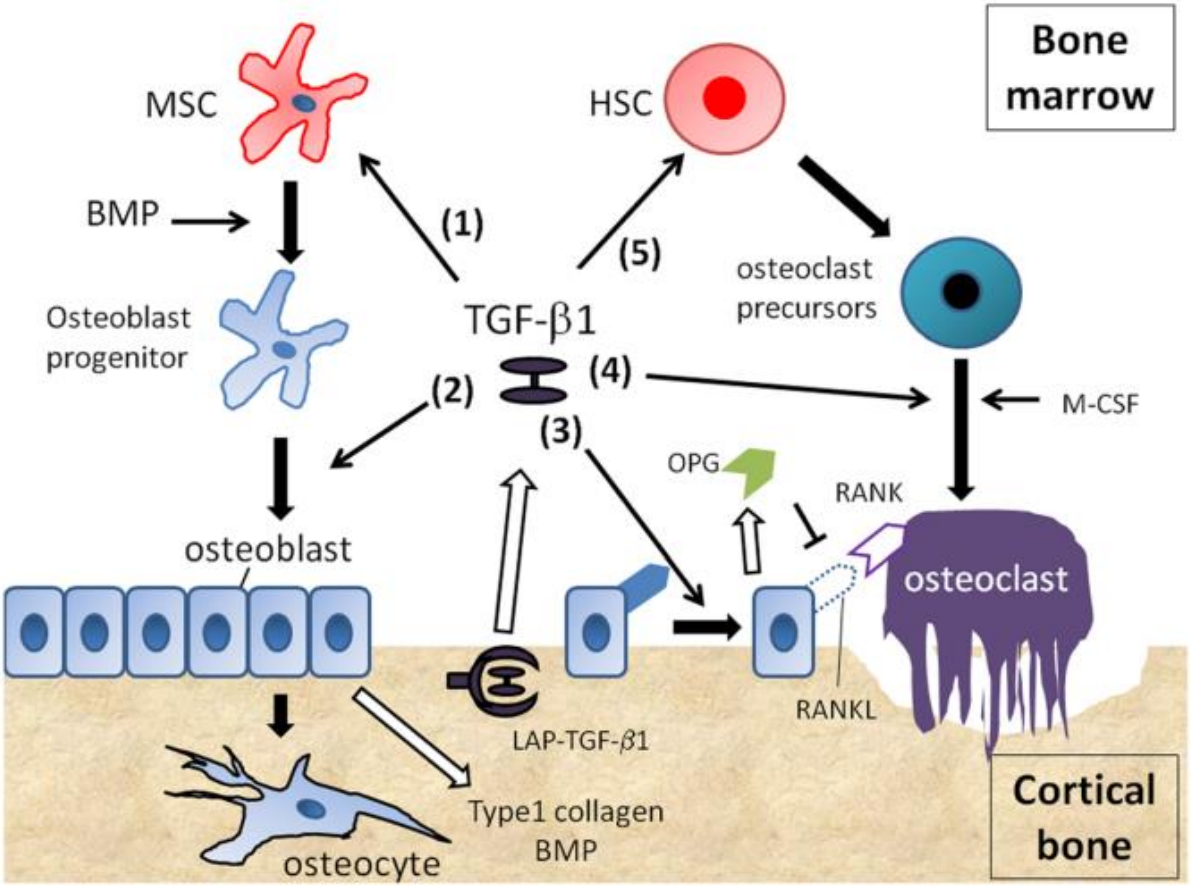


Figure 98: ***Five major role of TGF-β1 in osteoimmunity are shown****. (1) TGF-β1 stimulates the proliferation of MSCs, and promotes their differentiation into chondrocytes. (2) TGF-β1 promotes osteoblast progenitor's differentiation into osteoblast. (3) High concentration of TGF-β1 enhances osteoblast proliferation, and downregulates the expression of RANKL of osteoblast. (4) Low concentration of TGF-β1 promoted osteoclast maturation. (5) TGF-β keeps hematopoietic stem cells (HSC) in hibernation state. From Kasagi and Chen 2013.*

4. Geometrical versus biochemical signals

Finally, the ability to control cell shape is a useful aspect of micropattterning that could provide insight into the contribution of geometrical and biochemical cues on regulate stem

cell fate determination. In the context of the works described above, we mainly used square shapes, due to their physiological relevance and the fact that they are able to clearly highlight changes in cytoskeletal organization better than circle for example.

As shown when studying matrix remodeling, we are also able to generate patterns with different shapes, and in particular different aspect ratios. Rectangles of higher aspect ratios resemble more closely the aspect of a muscle fiber, whereas square shapes resemble the cuboidal shape of differentiated osteoblasts. As both types of patterns present the same overall area, seeding myoblasts or MSCs, which present a differentiation potential for both myogenic and osteogenic lineages, on patterns of these different shapes could reveal how cell shape regulates differentiation, independently of cell spreading.

Furthermore, these patterns could be used to decouple the effect of cell shape and biochemical cues on differentiation. An experiment investigating this could involve seeding myoblasts or MSCs on patterns of high aspect ratios, which would encourage myogenic differentiation (Bajaj et al., 2011). By using FN-bound BMP-2 to generate these patterns, we would provide the cell with a biochemical cue encouraging an osteoblastic commitment, but geometrical cues encouraging a commitment toward the myogenic lineage. Cell behavior under these conditions could reveal how a cell responds to contradictory signals to regulate its behavior.

Overall, the micropatterns we were able to generate in these works represent a versatile tool for the study of the mechanisms regulating the stem cell niche, at a single cell level. Due to the normalization of parameters like cell shape or spreading, they permit an analysis over a great number of cells, ensuring the statistical robustness of the results. In the context of the stem cell niche, specifically, these patterns permit the decoupling of the numerous signals influencing stem cell fate, and thus their study in a highly controlled environment.

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Personal information

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Place of birth: St Germain en Laye, France

Experience

- **Since October 2014**: Ph.D. student at the Institut Polytechnique de Grenoble (Grenoble INP), "Microcontact printing of growth factors on biomimetic films" (supervisors: Thomas Boudou and Catherine Picart)
  - Study of the effect of BMP-2 presentation mode on early osteogenesis
  - Effect of printing conditions on cytoskeletal organization
  - Force measurements of cellular tension using TFM

- **February 2014 – September 2014**: QA Support for General Electric HealthCare (LifeCare Solutions, UltraSound and Diagnostic Imaging):
  - quality assessment, trending and control ; development and implementation of a continual improvement process
  - training technicians on service record completeness and good documentation practices
  - four-fold increase in service record completeness

- **August 2013 – February 2014**: Internship with General Electric (LifeCare Solutions):
  - statistical analysis of defects
  - quality assessment

- **February 2012 – July 2012**: Research internship at Tufts University in Medford (Massachusetts):
  - setup and use of a Fluorescent Lifetime Imaging Microscopy (FLIM) system
  - image acquisition and data analysis using Matlab
  - observation and characterization of healthy, cancerous and precancerous cells; study of their metabolism based on changes in fluorescence lifetime

- **January 2011 – December 2012**: translation work for the Université de Technologie de Compiègne. Translation from French into English of an internet site destined to help students studying the properties, treatments and processes linked to various materials.

- **Summer 2010**: Research internship at the Université Claude Bernard Lyon 1, studying the organization and dynamics of biological membranes, and in particular the impact of a mitochondrial enzyme on the agglutination of phospholipids.

Education

- 2014: Biomedical engineering degree (Masters Level) at the Université de Technologie de Compiègne (UTC), specializing in biomaterials and biomechanics.
- 2008 - 2010: Biochemistry at the Université Claude Bernard, in Lyon, France, with honors.

Other

- Correction of the English of several scientific articles for professors at the Université Claude Bernard Lyon 1, and for a doctor in pharmacology at the hospital La Pitié-Salpêtrière in Paris.
- Computer skills: Word, Excel, PowerPoint, knowledge of Matlab, ImageJ and C programming language

Personal interests

- Rock climbing, travelling, piano

Languages

- Fluent French and English (completely bilingual) – TOEIC score: 990
- Good level of German (B2 level of the Common European Framework of Reference for Languages)
- Fair level of Spanish

Publications

- "Complementary effects of two growth factors in multifunctionalized silk nanofibers for nerve reconstruction", PLoS One, 2014
- "Stiffness-dependent cellular internalization of matrix-bound BMP-2 and its relation to Smad and non-Smad signaling", Acta Biomateralia, 2016
- "Signal mingle: Micropatterns of BMP-2 and fibronectin on soft biopolymeric films regulate myoblast shape and SMAD signaling", Scientific Report, 2017

Conferences

- GDR Physique de la Cellule au Tissu (Physics from the Cell to the Tissue), Rennes, France: oral presentation (2015)
- Namisceb (Nano and Micro Systems for Cell Biology), Nanosciences Foundation, Grenoble, France: poster presentation (2015 & 2016)
- 90th ACS Colloid & Surface Science Symposium, Harvard University, Cambridge (MA): oral presentation (2016)
- Gordon Research Conference, New Bedford (MA) – Signal Transduction through Engineered Extracellular Matrices: poster presentation (2016)
- Gordon Research Seminar, New Bedford (MA) – Signal Transduction through Engineered Extracellular Matrices: oral and poster presentation (2016)
- Annual Meeting of the American Society for Cell Biology, San Francisco (CA): poster presentation (2016)

Résumé

In vivo, les cellules souches résident au sein de niches, des microenvironnements extrêmement spécialisés qui leur permettent de réguler leur prolifération ainsi que leur différentiation en cellules matures et fonctionnelles. Ces microenvironnements sont caractérisés par des interactions cellules-cellules, la présence de facteurs de croissance et de cytokines, et une matrice extracellulaire, qui fournissent des signaux qui permettent de contrôler le comportement des cellules souches.

Bone morphogenetic protein 2 (BMP-2) est un facteur de croissance ostéoinductif qui est impliqué dans toutes les étapes de la différentiation ostéoblastique, ainsi que dans la transdifférentiation de myoblastes vers une lignée ostéogénique. In vivo, ce facteur existe à la fois en solution et lié à la matrice extracellulaire. Bien que ces deux modes de présentation influencent les comportements cellulaires de façon distincte, leur rôle dans le microenvironnement de la niche et leur pertinence fonctionnelle dans la genèse de la réponse biologique n'a presque pas été étudié à l'échelle cellulaire. Ici nous avons utilisé l'affinité naturelle de la BMP-2 pour la fibronectine afin de créer des micromotifs de BMP-2 de taille cellulaire sur des biomatériaux mous.

Cette technique nous a permis de contrôler simultanément la présentation spatiale de la BMP-2 liée à la fibronectine, ainsi que l'étalement cellulaire. Ces micromotifs ont induit une organisation d'actine et d'adhésions focales autour du noyau, et a déclenché la phosphorylation et la translocation nucléaire de SMAD1/5/8 dans des myoblastes murins C2C12 et des cellules souches mésenchymateuses. Ceci est un indicateur précoce de leur transdifférentiation ostéoblastique. Nous avons trouvé que l'étalement cellulaire lui-même facilitait la phosphorylation de SMAD1/5/8 dépendante de la BMP-2, et avons démontré que la signalisation SMAD médiée par la fibronectine et la BMP-2 dépendait de LIM kinase 2 et de ROCK, plutôt que d'une activation de la myosine II.

Nous avons également pu utiliser cet outil pour étudier les cinétiques d'adhésion et d'étalement, les changements d'organisation du cytosquelette dépendants du mode de présentation de la BMP-2, et la signalisation entre la matrice et la cellule, médiée par les intégrines. Nous avons obtenu des résultats préliminaires suggérant un effet du mode de présentation de la BMP-2 sur les forces cellulaires, suggérant que le mode de présentation des facteurs de croissance pourrait être pertinent à d'autres mécanismes cellulaires tels que la mécanotransduction.

Dans l'ensemble, nous résultats montrent que les patterns de BMP-2 liée à la fibronectine sont un outil utile à l'étude de mécanismes cellulaires. De façon plus large, notre approche pourrait être adaptée à d'autres combinaisons de protéines de la matrice et de facteurs de croissance, ouvrant ainsi une avenue fascinante pour recréer in vitro des niches spécifiques aux tissus.

Abstract

In vivo, stem cells reside within niches, highly specialized microenvironments which allow them to regulate their self-renewal and differentiation into mature and functional cells. These microenvironments are characterized by cell-cell interactions, the presence of growth factors and cytokines, and an extracellular matrix, all of which provide cues that control stem cell behavior.

Bone morphogenetic protein 2 (BMP-2) is an osteoinductive growth factor which is involved in all stages of osteoblastic differentiation, as well as the transdifferentiation of myoblasts toward an osteogenic lineage. In vivo, it exists both in solution and bound to the ECM. While these two modes of presentation are known to influence cell behavior distinctly, their role in the niche microenvironment and their functional relevance in the genesis of a biological response has sparsely been investigated at a cellular level. Here we used the natural affinity of BMP-2 for fibronectin (FN) to engineer cell-sized micropatterns of BMP-2 on soft biomaterials.

This technique allowed the simultaneous control of the spatial presentation of fibronectin-bound BMP-2 and cell spreading. These micropatterns induced a specific actin and adhesion organization around the nucleus, and triggered the phosphorylation and nuclear translocation of SMAD1/5/8 in C2C12 myoblasts and mesenchymal stem cells, an early indicator of their osteoblastic transdifferentiation. We found that cell spreading itself potentiated a BMP-2-dependent phosphorylation of SMAD1/5/8, and demonstrated that FN/BMP-2-mediated early SMAD signaling depended on LIM kinase 2 and ROCK, rather than myosin II activation.

We were also able to use this tool to investigate adhesion and spreading kinetics, changes in cytoskeletal organization depending on BMP-2 presentation mode, and reciprocal integrin-mediated signaling between the ECM and the cell. We were able to show preliminary results suggesting an effect of BMP-2 presentation mode on cellular forces, suggesting that growth factor presentation may be relevant to other cellular mechanisms like mechanotransduction.

Altogether, our results show that FN/BMP-2 micropatterns are a useful tool to study the mechanisms underlying BMP-2-mediated mechanotransduction. More broadly, our approach could be adapted to other combinations of ECM proteins and growth factors, opening an exciting avenue to recreate tissue-specific niches in vitro.