Presentation of BMP-2 from a Soft Biopolymeric Film Unveils its Activity on Cell Adhesion and Migration

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Cell biologists and biomaterials scientists are used to presenting cell adhesion ligands as they are in vivo: that is, in their insoluble form and in proximity to the cell adhesion site. Intriguingly, this approach has barely been adopted for growth factors, usually presented to the cell in solution or as a releasable molecule, whereas growth factors in vivo are known to strongly interact and to bind with the components of the extracellular matrix. In addition to these biochemical signals, the mechanical properties of the cell’s microenvironment are known to greatly impact cell behavior. To the best of our knowledge, the interplay between the mechanical properties of a biomaterial and the presentation of growth factor by the same material has not been investigated, to date.

Over the years, growth factors have been shown to govern numerous cellular processes and are now recognized as promising therapeutic tools. Many tissue-engineering efforts to date have focused on modifying surfaces with extracellular matrix (ECM) proteins or adhesive peptides. Growth factors have been mixed with biomaterials to be released by diffusion. In fundamental cell-biology research, there is also a large body of research dedicated to understanding the signalization pathways underlying cell stimulation by growth factors. Until now, in these cell biology studies, growth factors have mostly been added in solution to the culture medium of cells grown on stiff materials such as glass or tissue-culture polystyrene. The effects of such stiff materials on cellular processes can be quite subtle. Often, harsh serum starvation for about one day is necessary to render the cell quiescent, if any consequence of growth factor treatments on cellular behavior is to be observed. Interestingly, an interplay between cell adhesion-receptor (e.g., integrin) signaling and growth-factor signaling has also been evidenced by plating cells on a given type of ECM protein to induce a specific signal while providing the growth factor in solution. The concept of presenting a growth factor “in the solid state”, which we will, here, refer to as “matrix-bound”, has been proposed in the pioneering work by Griffith et al. on tethered epidermal growth factor (EGF), but it has, surprisingly, been neglected for a long time. However, this mode of delivery is closer to physiological conditions, since most growth factors in ECM are bound to proteins and glycosaminoglycans. Thus, they are thus presented to cells in a matrix-bound manner. Only recently, this concept has been extended to other types of growth factor, including the vascular endothelial growth factor (VEGF) and the insulin-like growth factor 1. These recent experimental studies showed that the efficacy of the growth factors was improved, compared to the soluble form, and that their biological function was enhanced.

The study of the family of bone morphogenetic proteins (BMPs) is an intensive field of research toward tissue-engineering applications and for fundamental insight into cell biology due to its physiological importance. BMPs play a crucial role in morphogenesis, tissue patterning, and regeneration after tissue damage, including cell differentiation. In particular, BMP-2 is a highly potent morphogen that induces muscle precursors and mesenchymal stem-cell differentiation in bone cells. Cell biologists have already noticed that BMP-2 added in solution to serum-starved cells plays also a role in early adhesive events, including adhesion and migration through actin reorganization. The delivery of matrix-bound BMP-2 in a bioactive form is still a major challenge in the field of tissue engineering one of the ultimate goals is to control cell differentiation in contact with a carrier material. New types of materials, such as materials mimicking natural ECM including fibrin films, polypeptide, polysaccharides-based layer-by-layer films, and hyaluronan hydrogels, appear particularly interesting. We have recently demonstrated that crosslinked poly(lysine)/hyaluronan (PLL/HA) polyelectrolyte multilayer films can effectively retain high and tunable quantities of human recombinant (rh)BMP-2. The effective bioactivity of BMP-2 was confirmed by measuring the osteogenic marker alkaline phosphatase that is expressed during the differentiation of muscle-derived C2C12 cells, which was directly related to the BMP-2 amount. We have also shown that myoblasts needed direct contact with the bound rhBMP-2 to start the differentiation process into bone cells, thus showing that BMP-2 was delivered in an immobilized fashion. In addition to chemical stimuli, the mechanical microenvironment of the cells is now recognized to play a key role in various aspects of cell processes, including adhesion, migration, and differentiation. Currently, synthetic polymers are the most popular tool for adjusting both biochemical and mechanical properties. However, natural materials are needed, again, and are emerging to mimic extracellular matrices by recapitulating...
several cues from the physiological cell microenvironment. After several cues from the physiological cell microenvironment. Using poly(L-lysine)/hyaluronan (PLL/HA) polyelectrolyte multilayer films of controlled stiffness, we recently demonstrated that film stiffness induces dramatic changes in C2C12 myoblast cell adhesion and spreading, and that myoblasts can differentiate into myotubes, with morphological differences depending on the stiffness of the film.

Here, we advantageously combine two functionalities of the biomimetic (PLL/HA) films, namely (i) the ability to modulate the film stiffness and (ii) the influence on the bioactivity of the film by presenting BMP-2 in a matrix-bound fashion (Figure 1) to investigate early cellular events. We focus on cell adhesion and migration, which are key steps prior to differentiation. Indeed, these processes are of utmost importance in embryonic morphogenesis, tissue regeneration, and tumor metastasis. We demonstrate that, by modulating both the presentation of the growth factor (soluble versus matrix-bound) and the mechanical properties of the material, it is possible to provide a permissive niche and unravel early cellular events related to confinement of BMP-2 at the ventral cell surface.

The design principle of the biomimetic nanoassembly is depicted in Figure 1. First, we deposited a layer-by-layer film of PLL and HA composed of 12 layer pairs (~1.3 μm in thickness) on a supporting material (glass substrate) (Figure 1A). Second, to control the film stiffness, we covalently crosslinked the films by using carbodiimide chemistry, as previously described. Two crosslinker concentrations were used, resulting in films with different Young moduli (Table S1 in the Supporting Information (SI)). Hereafter, these films will be referred to as soft and stiff films. Finally, we loaded the films with rhBMP-2 by post-diffusion in acidic condition, as previously described. Using this strategy, tunable amounts of BMP-2 can be loaded in the films, depending on the initial concentration of BMP-2 and the film thickness. We have previously shown that, after a slight release during the first hours, the amount of BMP-2 remained stable without any further loss of BMP-2 during the next four days. Thus, BMP-2 can be considered as matrix-bound. We verified that the amount of rhBMP-2 loaded by post-diffusion into these films did not vary significantly in the two types of film at our working concentration (20 μg mL⁻¹ for the initial rhBMP-2 concentration in solution, see Figure SI1 in the SI). Furthermore, we verified that BMP-2 loading did not affect the film’s overall morphology (Figure SI2), roughness, mechanical properties, or wettability (Table S1). This simple design strategy enables us to use a well-characterized biomimetic film that allows the variation of two major properties simultaneously: its stiffness and the presentation of matrix-bound BMP-2.

For the cell culture experiments, we chose C2C12 myoblasts as a working model. These cells constitute an acknowledged in vitro model system to study the ability of BMPs to alter cell lineage.
from the myogenic to the osteogenic phenotype. In a low concentration of serum, C2C12 cells differentiate into multinucleated myotubes. In contrary, BMP-2 treatment inhibits myotube formation and induces osteoblastic differentiation, as characterized by alkaline phosphatase activity or osteocalcin production. Importantly, these cells are also already known to be sensitive to the mechanical properties of the underlying matrix in terms of adhesion, spreading, and differentiation in myotubes.

C2C12 myoblasts were cultured on a stiff control surface (polystyrene culture plates and glass) and on soft and stiff BMP-2-loaded (PLL/HA) films containing bound BMP-2 (bBMP-2). Importantly, in the present work, the serum-starvation step was suppressed in order to test the ability of the bioactive films to induce specific cellular events in a complete medium and to investigate whether this effect is sustained.

Significant differences in the quantities of BMP-2 available to the cells might cast doubt on the conclusions regarding the biological importance of soluble versus bound BMP-2 presentations. Although it is very difficult to ensure that similar quantities are accessible by the cells in the case of soluble versus bound BMP-2, we ensure that the number of BMP-2 molecules surpassed the number of BMP-2 receptors in both conditions. We measured a bBMP-2 concentration of \( \approx 700 \text{ ng cm}^{-2} \) in the films (Figure S1), which corresponds to \( 3 \times 10^5 \) molecules per \( \mu \text{m}^2 \). Theoretically, this is enough to saturate the endogenous BMP-2 receptors (estimated at less than 100 per \( \mu \text{m}^2 \) cell surface area for cells typically expressing BMP2R at 10 000 receptors per cell). When delivered in solution (sBMP-2), a high concentration of BMP-2 was used (600 ng mL\(^{-1}\)) allowing the assumption that the receptors were saturated. This was supported by the fact that alkaline phosphatase expression showed a plateau for concentrations equal or higher than 300 ng mL\(^{-1}\) (Figure S3).

We first verified that bBMP-2 is effectively active when bound to soft and stiff films by performing two complementary assays. We measured the luciferase activity of C2C12 cells transfected with a BMP-responsive element (Id1) fused to the firefly luciferase reporter gene. We found that the luciferase expression was significantly increased in the presence of soluble and bound BMP-2 for all conditions. The luciferase signal of bBMP-2 on stiff films and sBMP-2 was similar, but it was significantly higher for bBMP-2 on soft films. In addition, we analyzed the phosphorylation of Smad, a protein known to play a key role in the transduction pathway from BMP-2 receptors to the nucleus. The expression of the phosphorylated form of Smad was measured by Western blot (Figure 1C). The level of Smad phosphorylation in the presence of sBMP-2 and bBMP-2 on stiff films was similar, but a higher phosphorylation was observed for bBMP-2 on soft films. Overall, this indicates that BMP-2 is bioactive in all conditions, with a similar or even enhanced Smad signal observed when it is delivered in a matrix-bound manner.

In the next step, we measured the effect of sBMP-2 or bBMP-2 on the initial cell adhesion and spreading. In the first 4 h of adhesion, only minor effects were observed for cells grown on plastic and stiff films but the number of adherent cells was significantly increased on soft films in the presence of bBMP-2. *p < 0.05.
after cell seeding, the cells spread rapidly on stiff standard tissue culture material ($≈3\,\mu\text{m}\,\text{min}^{-1}$) (Figure 2A) and on stiff films ($≈2\,\mu\text{m}\,\text{min}^{-1}$) (Figure 2A’). As cells spread already very fast on stiff substrates in the absence of BMP-2, it was not surprising to find that soluble or matrix-bound BMP-2 did not lead to a significant improvement on cell spreading kinetics on these stiff substrates. In contrast, the spreading kinetics were significantly enhanced in the case of soft (PLL/HA) films presenting bBMP-2 ($≈3\,\mu\text{m}\,\text{min}^{-1}$, Figure 2’) whereas cells remained round and poorly spread ($≈0.1\,\mu\text{m}\,\text{min}^{-1}$) in the absence of BMP-2. Interestingly, cells even remained round and poorly spread in the presence of sBMP-2 (Figure 2’). This specific induction of spreading on soft films by bBMP-2 was also associated with a considerable increase in the number of adherent cells after 4 h on soft films (Figure 2B). Cell attachment kinetics for bBMP-2 presented on soft (PLL/HA) films thus revealed that bBMP2 potentializes initial myoblast adhesion and spreading, whereas this inductor effect was totally masked in the case of stiff films. Our results suggest that adding sBMP-2 is not efficient in our culture conditions, regardless of the substrate. Moreover, the effect of bBMP-2 is specific and is not related to the presence of serum in the culture medium, since the striking effect of bBMP-2 on soft films was still observed in a serum-free medium (Figure S4). Furthermore, the effect of bBMP-2 cannot be attributed to the minor changes of film stiffness upon addition of bBMP-2 (Table S1), as these changes are not statistically significant. Indeed, they were not sufficient to induce any significant change in cell spreading for other cells, such as NIH3T3 fibroblasts (Figure S7).

Continuing our analysis of cell adhesion, we investigated whether the effect of BMP-2 was sustained for several hours. We thus observed cell morphology using fluorescence microscopy after staining the actin cytoskeleton and measured the cell spreading areas for all experimental conditions at a longer time scale, after 16 h of culture (Figure 3). After initial adhesion, the trends remained the same. On the control tissue culture polystyrene surface (TCPS) surfaces, cells spread considerably, both with and without sBMP-2 (Figure 3A, upper row). Similarly, the cells on the stiff films exhibited a high spreading area that was not changed by bBMP-2 (Figure 3A, middle row). Only cells cultured on soft films with bBMP-2 highlighted the drastic effect of BMP-2 on the increase of cell adhesion and spreading (Figure 3A, lower raw). We also observed that the focal adhesion protein vinculin exhibited a diffuse fluorescence for cells on soft films with no evidence of focal adhesion formation (Figure S15). Conversely, organization of focal adhesions correlated with the spreading of cells grown on bBMP-2-soft films. This observation suggests that integrins are activated and clustered during the cell response to matrix-bound BMP-2.

It is important to note that our findings are not specific to C2C12 myoblast cells. Indeed, we found that MC3T3-E1 osteoblast precursor cells, an acknowledged cellular model to study differentiation in bone cells[38] and which also possess BMP2 receptors, responded similarly. Their adhesion and spreading were drastically increased using bBMP-2 on soft films (Figure S6). Importantly, this spreading induced by BMP-2 signals is specific to BMP-2-receptor-expressing cells that respond to BMP-2 signals, since we did observe only a very minor effect on NIH3T3 cells, known to express some BMP-2 receptors,[39] but known not to transduce BMP-2 signals[39] (Figure S7).
Stiffness of a matrix, on one hand, and BMP-2 stimulation, on the other hand, have already independently been shown to play a key role in cancer metastasis.[18] In addition, BMP-2 has been described to enhance cell migration in several cell lines. Therefore, we further addressed whether the cell migration on the different surfaces was also affected by sBMP-2 or bBMP-2 (Figure 4). Once again, in our culture conditions (complete medium and no preliminary starvation step applied to cells), sBMP2 had no significant effect on cell migration on any types of surface. In contrast, bBMP-2 significantly increased cell migration on stiff films and in an even greater extent on soft films. In summary, our findings indicate that bBMP-2 had a rapid, significant, and sustained effect on the dynamics of the actin cytoskeleton in C2C12 cells, which can be specifically attributed to the presentation of the growth factor by the soft film, as opposed to the soluble presentation of BMP-2.

Now, a key question is: how can this effect of bound BMP-2 be interpreted? We hypothesize that these dramatic changes in cell shape and migration speed are likely consequences of combined parameters, which are due to the reduction of dimensionality, from BMP-2 presentation in 3D to its presentation in 2D. This spatial confinement leads to: i) its restricted diffusion, as it is bound to the film and not freely diffusing; ii) its increased availability, that is, when a BMP-2 ligand is disengaging, another ligand is available in close proximity; and iii) an interplay of BMP-2 receptors with other types of receptor such as cell-adhesion receptors. The differences between matrix-bound and solution presentation of BMP-2 are summarized in Figure 5.

This spatial confinement is especially important for the kinetics of the BMP-2 receptor/ligand interactions. Indeed, BMP-2 is dimeric in nature and can bind multiple BMP-2 receptors.[10] Furthermore, BMP-2 receptors (BMP2R), which are mainly composed of type I (BMP2R-I) and type II (BMP2R-II) receptors, can form oligomers. At the cell surface, multiple forms of pre-assembled receptor oligomers exist, independently of the ligand’s presence, under heterodimeric complexes consisting of type II receptor and type I receptors.[21] According to Nickel et al.,[40] who deeply investigated the binding kinetics of different BMP receptors with BMP-2, 90% of the total binding sites are low affinity sites (1–4 μM), which most likely reflect receptor monomers, whereas only 10% of the binding sites exhibit high affinity binding (0–500 pM). Thus, the occupancy rate of the low affinity receptors may be greatly enhanced in the presence of a large amount of bBMP-2 molecules, which are characterized by both a 2D presentation (spatial confinement) and restricted diffusion. This cooperative binding via multiple interactions is expected to enhance the occupancy rate of BMP-2 receptors at the cell surface.

Figure 4. Matrix-bound BMP-2 potentiates cell migration. Migration velocity (μm h⁻¹) of C2C12 cells cultured on different substrates: standard tissue culture polystyrene (TCPS) and either stiff or soft films. Matrix-bound bBMP-2 significantly increases the cell velocity on stiff films and even more significantly on soft films. The soluble form of BMP-2 had no significant effect compared to the condition where no BMP-2 is added, except on TCPS. *p<0.005

Figure 5. BMP-2 presentation from the ECM mimetic film allows spatial confinement of receptor complexes and BMP-2 ligand at the ventral side of the cell. This scheme summarizes the major differences between matrix-bound and soluble delivery of BMP-2 to the cell. When BMP-2 is bound to the film (left part of the scheme, 2D presentation), it is spatially confined and its diffusion is restricted. In addition, the occupancy rate of BMP-2 receptors is enhanced with a possible formation homo and heterodimeric receptor complexes and ligand/receptor binding is not limited by diffusion (a high number of free ligands is available in proximity of the receptors). Furthermore, due to the close proximity of growth factor receptors and adhesion receptors, a crosstalk between these two types of receptor is possible. Thus, cross-talks between BMP-2 signaling and adhesion signaling, which can induce cytoskeleton remodeling, might explain the striking effects observed for cells plated on soft films with bBMP-2. Such cooperative effect cannot be observed when BMP-2 is presented in solution (right part of the scheme, 3D presentation), i.e., BMP-2 can freely diffuse in 3D and has a low availability due to the diffusion-limited reaction between receptors and ligands. Furthermore, in this case, BMP-2 receptors are diffusing at the plasma membrane and are not at the vicinity of adhesion receptors.
interactions between ligand and receptors, called avidity, could strongly enhance the clustering of the BMP-2 receptors. Furthermore, the confinement of BMP-2 at the ventral cell surface and the striking effect of BMP-2 on cell shape and cytoskeletal dynamics suggest cooperation between growth factor receptors and adhesion receptors. Such cooperation between adhesion receptors and growth factor receptors has never been evidenced for BMP-2 receptors. Particularly interesting candidates are integrins, which are known mechano-sensors. Indeed, it has already been shown that the mechanical link between integrins and the ECM can be enhanced by cis-signaling from growth factors and other ligands. In integrins, which are known mechano-sensors. Indeed, it has already been shown that the mechanical link between integrins and the ECM can be enhanced by cis-signaling from growth factors and other ligands.

Conversely, one may speculate that the ligand itself (TGF-β) interacts with integrins in a signaling platform that would activate both BMP-2R and integrin receptors, or it might activate integrin receptor through its binding to BMP-2 receptor. BMP-2 receptor may thus behave as “ligand density sensors”, similarly to what has been evidenced for ephrinB1 receptors, which modulate integrin-mediated cell-matrix attachment according to the density of the ephrinB1 ligands they encounter. This hypothesis is supported by recent work that evidenced that tethered EGF can act in synergy with adhesion ligands on soft synthetic hydrogels. Finally, BMP-2 may be prevented from internalization, and this may trigger different, longer lasting signaling events compared to sBMP-2. These hypothesis need to be further investigated in the future.

This “matrix-bound” mode of delivery thus shows the importance of spatial organization of ligand and their receptors, restricted diffusion of ligand, and possible subsequent receptor clustering at cell membranes. The adhesive and migratory response suggests that ECM-bound BMP-2 could act to organize complexes of receptors in the plane of the plasma membrane, and it highlights the requirement for juxtaposition of two receptors (growth factor and adhesion receptors) to trigger a specific signaling pathway.

Altogether, these results imply that the materials used for cell culture should be chosen with great care when studying the effects of growth factors on cells, given that the properties of the material alone on the cellular behavior are far from negligible. Certain materials can induce spontaneous cellular differentiation, apoptosis, and most standard cell-culture materials are designed to enhance cell adhesion and spreading. There is a need to develop new materials offering the status of a “permissive” niche, i.e., an environment in which the cells are stimuli-responsive and not driven to a specific response due to the underlying stiff substrate. We designed a highly tunable material that halts cell spreading from the outset, while retaining cell viability and maintaining their potential for adhesion and migration when the proper mechanism is engaged. A population of what could be named “adhesion-competent” cells, i.e. adherent, viable, and able to spread and migrate upon stimulation, can thus be obtained when cells are grown on soft films. These adhesion-competent cells can be of great use for unraveling the subtle effects of various chemical or physical cues on cell adhesion and spreading. These multifunctional biomimetic films have enabled us to clearly evidence the effects of biochemical signals triggered by matrix-bound BMP-2, unperceived when cells are plated on a stiff matrix.

In summary we have developed a biomaterial offering the characteristics of a permissive niche. For the first time, we have been able to show, using C2C12 myoblasts as model BMP-2 responsive cells, that cells behave differently when BMP-2 is presented in matrix-bound form on a soft matrix, rather than in its soluble form. This was proven here for early cellular events. The exact mechanism for these differences is currently unknown and requires further studies of the dynamics of BMP-2 signaling pathways (sBMP-2 versus bBMP-2). Of particular interest are BMP-2 receptor/integrin cooperation, cytoskeletal dynamics, biochemical signaling pathways, differentiation, and the interplay between matrix mechanical properties and growth factor stimulation. Our results indicate that signaling can be enhanced by the spatial organization of different types of receptors and that receptor cooperativity is necessary for the achievement of a coordinated cell response. We anticipate that this concept of presenting matrix-bound growth factors and bioactive molecules from films of tunable stiffness will be used to answer fundamental biological questions on growth-factor signaling, and that they will lead to the development of a new generation of functional biomaterials.

**Experimental Section**

**Preparation of Films, Cross-Linking, and Loading of rhBMP-2:** HA (sodium hyaluronate, \(2 \times 10^{-6} \text{ g mol}^{-1}\)) was purchased from Lifecore Biomedical (USA) and PLL (2 \(10^{-6} \text{ g mol}^{-1}\)) was purchased from Sigma (France). PLL (0.5 mg mL\(^{-1}\)) and HA (1 mg mL\(^{-1}\)) were dissolved in a Hepes-NaCl buffer (20 mM Hepes, 0.15 v NaCl, pH 7.4). For all the cell experiments, the films were prepared, as previously described, with a dipping machine (Dipping Robot DR3, Kierstein GmbH, Germany) on 14 mm diameter glass slides (VWR Scientific, France). For the cell adherence study, the films were manually constructed in 96-well plates (Nunc, Denmark) starting with a first layer of poly(ethyleneimine) (7 \(10^{-6} \text{ g mol}^{-1}\), Sigma, France) at 3 mg mL\(^{-1}\). Briefly, polyelectrolyte solutions (50 μL) were deposited in each well, left to adsorb for 8 min, before being washed twice with rinsing solution (100 μL of 0.15 v NaCl, pH 6) for 1 min. The sequence was repeated until the build-up of (PLL/ HA)\(_{12}\) was achieved (12 is the number of layer pairs). The films were crosslinked following the protocol previously described using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) at 30 (soft films) or 100 mg mL\(^{-1}\) (stiff films) and N-hydroxysulfosuccinimide (sulfo-NHS) at 11 mg mL\(^{-1}\) (both purchased from Sigma, France).

The BMP-2 (Clinical Grade, Wyeth BioPharma, USA) was incorporated into films pre-equilibrated for 30 min in the medium in which BMP-2 was suspended (HCl 1mM). A volume of 50 μL for 96-well plates or 0.3 mL for 24-well plates of BMP-2 was deposited on to the films and left to adsorb overnight at 4°C. 150 μL of Hepes-NaCl were then added to each well and left at room temperature for 15 min. The coated slides were thoroughly washed for 7 h in Hepes-NaCl in order to keep only matrix-bound BMP-2, before being sterilized for 15 min under UV light. The experiments were carried out at least in triplicate, with three independent samples per condition in each experiment.

**Cell Culture:** C2C12 cells (<20 passages) were maintained in polystyrene flasks in a 37°C, 5% CO\(_2\) incubator, and cultured in a 1:1 Dulbecco’s modified eagle medium (DMEM)/F12 medium (Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France), containing 10 U mL\(^{-1}\) penicillin G and 10 μg mL\(^{-1}\) streptomycin (Gibco, Invitrogen, Cergy-Pontoise, France) (growth medium, GM). Cells were subcultured...
prior to reaching 60–70% confluence (approximately every 2 days). For all experiments, C2C12 cells seeded on films at 1.5 × 10^6 cells cm^−2 in growth medium were allowed to grow for one day.

Quantification of Cell Adhesion and Spreading: The cell counting tests were performed in 96-well plates. The cell numbers were assessed after 4 h of adhesion using a cell-counting kit (CyQUANT, Molecular Probes, Invitrogen, Cergy-Pontoise, France). In brief, the cells were washed three times with phosphate buffered saline (PBS) and frozen at −80 °C overnight. After thawing the cells at room temperature (RT), a mixture of the CyQUANT GR dye and cell-lysis buffer was introduced, and the fluorescence of the plates was measured using the Tecxan Infinite 1000 spectrophuorometer (Ex 485/Em 535) (Tecan, Austria).

For F-actin staining, the cells were fixed in 3.7% formaldehyde in PBS for 20 min and permeabilized for 4 min in TBS (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.2% Triton X-100. Slides were blocked in TBS containing 0.1% BSA for 1 h, and subsequently incubated with rhodamine-phalloidin (1:800) in TBS with 0.2% gelatin for 30 min. For focal adhesion staining, anti-vinculin (1:400, Sigma V9131) antibodies in Tris buffered saline with 0.2% gelatin were incubated for 30 min. AlexaFluor488-conjugated secondary antibody was then incubated for 30 min.

All the slides were mounted onto coverslips with antifade reagent (Prolong) and viewed with fluorescence microscopy (Axiovert 200M, Zeiss, Germany) using 20× or 63× apochromatic objectives. Images were acquired with Metaview software using a CoolSNAP EZ CCD camera (both from Roper Scientific, Evry, France). To quantify cell adhesion and spreading, fluorescence images were analyzed with the ImageJ software (v1.42, NIH, Bethesda) to determine average cell numbers and cell area (in μm²).14,19

Smaβ Assay Using Luciferase Reporter Gene and Western Blots: C2C12-A5 were stably transfected with an expression construct (BRE-Luc) containing a BMP-responsive element fused to the firefly luciferase reporter gene.17 They were cultured in same conditions as C2C12 cells. After one day of culture on the substrates, cell lysis and luciferase measurements were carried out according to the manufacturer’s instructions (Bright-Glo detection system, Promega). Measurements were carried out according to the DNA content of each sample as measured by the CyQUANT assay.

Detection of phospho-Smads by Western blotting was done according to standard protocols. After electrophoresis and transfer (10 μg Tris, pH 7.9, 150 μm NaCl, 0.5% Tween 20, 3% dry milk at room temperature for 1 h), the membrane was incubated with anti-Phospho Smad1/5/8 (cell signalling, 1:1000 dilution) overnight at 4 °C. Detection of adsorbed antibodies was performed by ECL (Amershams Biosciences, Inc.), after incubation with horse-radish peroxidase, diluted 1:13 000. As a control, detection of actin was also performed.

Time Lapse Image Acquisition: Time lapse images were acquired over a 20 h period on either标准 polystyrene culture plates, soft or stiff growth medium were allowed to grow for one day.

Statistical Analysis: All experiments were repeated at least three times. Error bars represent standard errors, and statistical analysis was performed using the one-way analysis of variance test (ANOVA) to evaluate the statistical differences (p < 0.05 or p < 0.005) among all samples or between samples and controls, respectively.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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