C₂C₁₂ myoblast/osteoblast transdifferentiation steps enhanced by epigenetic inhibition of BMP2 endocytosis

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In this study, we investigated the role of endocytosis in cytokine-dependent gene transcription events leading to cell differentiation by analyzing BMP2-dependent critical transcriptional steps of the myoblast/osteoblast transdifferentiation in C₂C₁₂ cells (2, 11), in response to chemically (18, 20) or mechanically (16) mediated epigenetic inhibition of bone morphogenetic protein 2 (BMP2) endocytosis. Crude modulations in the dynamics and amplitude of critical BMP2-dependent transcriptional steps of the myoblast/osteoblast transdifferentiation were observed in specific response to both treatments inhibiting endocytosis. These results suggest that epigenetic modulation of endocytotic internalization of the ligand-receptor complex and cytokine-mediated effects on the transcription of genes controlling critical physiological events, such as cell differentiation, remains largely uninvestigated.

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MATERIALS AND METHODS

Cells and materials. The C₂C₁₂ mouse pluripotent mesenchymal cell line was grown in DMEM with L-alanylglutamine

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Cells were factor of 0.7 during the endocytosis experiment. Trypan blue FBS rendered hyposmotic by dilution in distilled water by a factor of 0.7. Cells were incubated in 4% paraformaldehyde for 10 min at 37°C. They were washed three times in TNT and were incubated for 30 min with 3% H2O2 in PBS for 30 min at room temperature to inhibit endogenous peroxidase activity. Thereafter, we incubated cells for 45 min at 37°C in fresh DMEM supplemented with 0.5% BSA (TNB blocking buffer) provided by Satoshi Sato (Kyoto University). Speciﬁcation and labeling of the BMP2–primary antibody complex with an appropriate secondary antibody, as described below. Note that the anti-BMP2 antibody might dissociate from BMP2 after internalization into lysosomes. In any case, endocytic internalization of exogenous BMP2 remains necessary to observe internal cytosolic labeling of the cell. The use of Triton X-100, necessary to label the internalized BMP2, perturbs the fine structures of the plasma membrane and of cytoplasmic compartment membranes, inducing diffuse labeling of these structures.

Exogenous BMP2 internalization was analyzed quantitatively following the same protocol, except that the BMP2–anti-BMP2 antibody complex was labeled with the secondary antibody without Triton X-100. This ensured that only the BMP2 retained on the plasma membrane was ﬂuorescently labeled. Thus the cells were incubated in PEG-Chol–cyclodextrin medium before incubation at 4°C with exogenous BMP2 complexed with the corresponding speciﬁc primary goat antibody diluted in the same medium to facilitate the binding of the BMP2–primary antibody complex to its speciﬁc plasma membrane receptor. Cells were incubated from 0 to 45 min at 37°C in BMP2–free medium supplemented with PEG-Chol–cyclodextrin. After ﬁxation, labeling, and ampliﬁcation as described below, without Triton X-100, the cells were released in the volume. Fluorescence intensity was determined with a Fluoromax Jobin and Yvon spectrophluorometer at the ﬂuorescein isothiocyanate excitation wavelength of 408 nm and emission wavelength of 508 nm and indicated the amount of exogenous BMP2 that was not internalized via the plasma membrane. We deduced the amount of exogenous BMP2 taken up by comparison of the ﬂuorescence intensity at the end of the experiment with that at time 0.

RESULTS

Endocytotic internalization of exogenous BMP2 is inhibited by PEG-Chol–cyclodextrin treatment. We investigated the endocytosis of exogenous BMP2 in the presence of a mixture of PEG-Chol, which inhibits bulk ﬂow- and caveola-dependent endocytosis (8), and cyclodextrin, which inhibits clathrin- and caveola-dependent endocytosis (18, 20).

Quantitative observations showed considerable cytoplasmic internalization of BMP2 within 4–10 min at 37°C (Fig. 1Ab), whereas no detectable cytoplasmic internalization of BMP2 was observed in the treated cells after 10 min at 37°C (Fig. 1Ac). Quantitative
spectrofluorimetry showed a very efficient internalization of the BMP2 within 2 min, consistent with the time scale of clathrin-dependent receptor-mediated endocytosis (6). On the other hand, the BMP2 internalization was strongly inhibited in treated cells over a 45-min time scale. The endocytosis of exogenous BMP2 was completely blocked for the first 10 min, and the mean amount of plasma membrane BMP2 internalized was 3.7 times higher in untreated than in treated cells after 45 min (Fig. 1B).

Endocytotic internalization of exogenous BMP2 is inhibited by hyposmotic treatment. We blocked the endocytotic internalization of exogenous BMP2 mechanically by simply subjecting cells to a hypotonic shock in BMP2-free medium at 37°C (16) for 0–45 min after incubation with the BMP2-primary antibody complex at 4°C. We found that exogenous BMP2 internalization in adherent C2C12 cells was inhibited by a decrease in external osmotic pressure by one-third. No detectable cytoplasmic internalization of BMP2 was observed in the first 10 min after osmotic treatment (Fig. 1A4).

Quantitative analysis confirmed that BMP2 endocytosis was blocked during these first 10 min. Over 45 min, the level of BMP2 internalization was found to be 3.4 times higher in untreated than in treated cells (Fig. 1B).

Note that BMP2 was also labeled with its specific antibody after fixation rather than prelabeled before the endocytosis process. The same endocytic internalization fates as in Fig. 1A were observed, despite the presence of the endogenously expressed BMP2 that introduced noise in the observations (data not shown). This ensured that the endocytic internalization fate of prelabeled exogenous BMP2 monitors the endocytic internalization rate of unlabeled exogenous BMP2.

Finally, quantitative analysis showed no significant change in the concentration of exogenous BMP2 on the plasma membrane at time 0 of the experiment in response to both chemical and physical perturbations (Fig. 1C).

Smad1 nuclear translocation is strongly accelerated by inhibition of the endocytotic internalization of exogenous BMP2. We investigated the dynamics of Smad1 nuclear translocation after inhibition of the endocytosis of exogenous BMP2. We found that Smad1 translocation to the nucleus was initiated at 30 min in the presence of exogenous BMP2 in untreated cells (Fig. 2b), as already reported (12, 14). After the chemical inhibition of BMP2 endocytosis with PEG-Chol-cyclodextrin, Smad1 massively translocated to the nucleus at only 3 min (Fig. 2d). Smad1 translocation was...
was therefore 10 times faster if the endocytosis of exogenous BMP2 was specifically inhibited by chemical factors or if physical factors had a mechanical effect on the plasma membrane cells.

The chemical inhibition of BMP2 endocytosis mediated by PEG-Chol-cyclodextrin accelerated and strongly increased JunB expression (Fig. 3, Ac and Ad). JunB expression was first detected after 10 min of incubation in treated cells, as opposed to 30 min in untreated cells (Fig. 3, Ba and Bb). After 12 min, the level of JunB expression in treated cells was identical to the maximal levels observed after 45 min in untreated cells. Moreover, JunB expression was maximal after 30 min in treated cells, rather than after 45 min, as observed in untreated cells. Furthermore, the peak of JunB expression in treated cells was three times higher than in untreated cells. Finally, the total amount of JunB produced within 60 min was four times higher in cells deficient in endocytosis than in endocytosis-competent cells (Fig. 3Bb). No JunB transcription was observed within 60 min of chemical treatment in the absence of BMP2 (Fig. 3, Ac and Bd).

Similar behavior was observed in response to physical inhibition of the endocytosis of exogenous BMP2 by hyposmotic treatment (Fig. 3, Ae and Af). JunB protein levels were maximal after 30 min in treated cells rather than after 45 min, as observed in untreated cells, and the peak was seven times higher in treated than in untreated cells. The total amount of JunB produced within 60 min was three times higher in treated cells, given the arbitrary shape assumed for the peak of JunB expression at 30 min (Fig. 3, Ba and Bc). No JunB transcription was observed within 60 min of hyposmotic treatment in the absence of BMP2 (Fig. 3, Ae and Be). Conversely, a slight decrease in background JunB expression was observed, suggesting slight degradation of JunB in response to hyposmotic shock (Fig. 3Be). This slight decrease may account for the much narrower peak of JunB expression after hyposmotic treatment in the presence of BMP2 than after chemical treatment (Fig. 3Bc).

Thus JunB levels after 60 min are four and three times higher, respectively, after inhibition of the endocytosis of exogenous BMP2 by chemical factors or physical factors with mechanical effects.

ALP and OPN expression levels are increased by inhibition of the endocytotic internalization of BMP2. The synthesis of ALP expression is one of the critical transcriptional responses of the cell induced by BMP2, indicative of osteoblast differentiation (10, 15). Chemical inhibition of the endocytosis of BMP2 with PEG-

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**Fig. 2.** Strong acceleration of the nuclear translocation of mother against Dpp 1 (Smad1) resulting from inhibition of the endocytotic internalization of BMP2 under PEG-Chol-cyclodextrin and hyposmotic treatments. Smad1 is preferentially located in the cytoplasm in the absence of BMP2 and treatment (see Ref. 11). a: no nuclear translocation of Smad1 was observed in the absence of treatment, after 3–20 min in the presence of BMP2 (+, BMP2; −, PEG-Chol/Cd, −, hyposmotic treatment; time <20 min). b: nuclear translocation of Smad1 was observed only after 30 min of incubation with BMP2. c: no nuclear translocation of Smad1 was observed in the absence of BMP2 in cells treated with PEG-Chol-cyclodextrin from 3 to 60 min. d: high levels of Smad1 nuclear translocation were observed after 3 min in the presence of BMP2 in cells treated with PEG-Chol-cyclodextrin. e: no nuclear translocation of Smad1 was observed in the absence of BMP2 in cells treated by hyposmotic shock from 3 to 60 min. f: high levels of Smad1 nuclear translocation were observed after 3 min in the presence of BMP2 in cells treated by hyposmotic shock. Note that degradation of the Smad1 translocated was also found to be very rapid and was observed after 5 min only. Each experiment was reproduced independently and successfully at least 3 times.
Fig. 3. A: increase in JunB expression resulting from inhibition of BMP2 endocytosis under PEG-Chol-cyclodextrin and hyposmotic treatments. a: background level of JunB expression in the absence of BMP2 and treatment (−, BMP2; −, PEGChol/Cd; −, treated treatment; time <60 min). b: JunB levels after 45 min of incubation with BMP2, without treatment. c: JunB levels from 10 to 45 min in the absence of BMP2 under PEG-Chol-cyclodextrin treatment. d: JunB levels after 30 min in the presence of BMP2 under PEG-Chol-cyclodextrin treatment. e: JunB levels from 10 to 45 min in the absence of BMP2 under hyposmotic treatment. f: JunB levels after 30 min in the presence of BMP2 under hyposmotic treatment. Each experiment was reproduced independently and successfully at least 3 times. Ba–Bc: quantitative analysis of the dynamic profile of JunB expression in the presence of BMP2, without treatment (a), under PEG-Chol-cyclodextrin treatment (b), and under hyposmotic treatment (c). Each point represents at least 2 experiments, except the 30-min experiment of Bc, which was reproduced independently 5 times to verify the reproducibility of the single “responsive point” of Bc. “JunB expression level” is the JunB expression quantity of treated cells in the presence of BMP2 minus the JunB expression quantity of untreated cells in the absence of BMP2 control, normalized to this control. Each point is the mean result of 2 experiments. Bd and Be: quantitative analysis of the dynamic profile of JunB expression is shown in the absence of BMP2 under PEG-Chol-cyclodextrin treatment (d) and under hyposmotic treatment (e). Each point represents at least 2 experiments. “JunB expression level” here is the JunB expression quantity of treated cells in the absence of BMP2 minus the JunB expression quantity of untreated cells in the absence of BMP2 control, normalized to this control. Each point is the mean result of 2 experiments; error bars of each quantitative experiment indicate fluctuations of the mean result.
Chol-cyclodextrin resulted in ALP levels five times higher than those of endocytosis-competent cells. Physical inhibition of the endocytosis of exogenous BMP2, by hypotonic shock, resulted in ALP levels 3.5 times higher than those of endocytosis-competent cells (Fig. 4A). Moreover, if endocytosis was chemically inhibited with PEG-Chol-cyclodextrin at the below-critical threshold BMP2 concentration of 50 ng/ml, cells synthesized ALP in similar amounts to those observed in cells incubated with 300 ng/ml BMP2 without inhibition of endocytosis (Fig. 4B). A similar effect was observed with hyposmotic treatment, but ALP levels are significantly lower than those observed for chemical inhibition (Fig. 4B). No increase in ALP levels in response to chemical or hyposmotic treatments was observed in the absence of BMP2 (data not shown).

OPN expression, another critical transcriptional step induced by BMP2 indicative of osteoblast differentiation (19), was investigated within the same experimental conditions at a BMP2 concentration of 300 ng/ml. Although significant OPN expression could not yet be detected in BMP2 endocytosis-competent cells under these experimental conditions, chemical inhibition with PEG-Chol-cyclodextrin and physical inhibition by hypotonic shock of the endocytosis of BMP2 both resulted in the trigger of OPN synthesis (Fig. 5). Again, hyposmotic treatment effects were significantly lower than effects observed for chemical inhibition of endocytosis, similar to effects on osteoblastic ALP expression levels. No increase in OPN levels in response to chemical or hyposmotic treatments was observed in the absence of BMP2 (data not shown).

Myogenin repression is amplified by inhibition of the endocytic internalization of BMP2. Myogenin repression is one of the critical transcriptional events in the response of the cell to BMP2, indicative of terminal myoblastic differentiation inhibition (9).

Chemical inhibition of the endocytosis of BMP2 with PEG-Chol-cyclodextrin resulted in the amplification of the myogenin repression of a factor of three compared with myogenin repression in the presence of BMP2 in endocytosis-efficient cells. Physical inhibition of the endocytosis of exogenous BMP2 by hypotonic shock also resulted in repression of myogenin levels three times higher than myogenin repression effects in endocytosis-competent cells (Fig. 6). No decrease in myogenin levels in response to chemical or hyposmotic treatments was observed in the absence of BMP2 (data not shown).

DISCUSSION

Inhibition, by chemical or physical means, of the endocytosis of exogenous BMP2 led to an increase in critical transcriptional responses involved in myoblast/osteoblast transdifferentiation of C2C12 cells.

In both cases, treatment increased the rate of nuclear translocation of the BMP2-dependent Smad1 transcription factor by a factor of 10. Interestingly,
Smad1 was concentrated at the nucleus border with an important residual pool remaining in the cytoplasm at initiation of Smad1 nuclear translocation after 30 min of BMP2 incubation without treatment. In contrast, Smad1 was highly concentrated within the nucleus and around the nucleus, with a poor cytoplasmic pool after 3 min of treatment inhibiting endocytosis, indicating a massive nuclear translocation of Smad1 in response to the inhibition of BMP2 endocytosis.

Consistent with this, Smad1-dependent JunB gene expression was increased by a factor of three to four over 60 min, and the maximum level of JunB protein was three to four times higher in treated cells. None of these effects were observed if endocytosis was inhibited in the absence of BMP2, demonstrating that the rate of Smad1 translocation and the level of JunB expression were increased specifically by inhibition of the internalization of BMP2. The interaction of BMP2 with its specific receptor is associated with the phosphorylation of the Smad1 transcription factor, leading to the binding of Smad1 to Smad4, triggering the translocation of Smad1 to the nucleus (7, 24). The induction of JunB transcription is the first transcriptional event initiating inhibition of myoblast differentiation to be triggered by the Smad1 transcription factor (5). Thus these results show that the transcriptional initiation of the inhibition of C2C12 myoblast differentiation is strongly amplified by inhibition of BMP2 endocytosis.

In addition to the JunB expression level increase amplifying inhibition of myoblastic differentiation initiation, the important increase in the dynamics of the BMP2-dependent nuclear translocation of the Smad1 transcription factor should amplify osteoblastic differentiation by increasing ALP expression levels (15). We effectively observed an increase by a factor of 3.6 to 5 in the expression of the characteristic osteoblastic marker ALP 17 h after the chemical or physical treatments in the presence of BMP2 for 60 min. Interestingly, cells incubated under the same conditions with below-critical threshold (50 ng/ml) concentrations of BMP2 showed no significant ALP synthesis within endocytosis-efficient conditions but recovered a synthesis of ALP after inhibition of BMP2 endocytosis. Thus inhibition of BMP2 endocytosis not only enhances the ALP expression levels characteristic of osteoblastic differentiation within normal BMP2 conditions but also triggers osteoblastic ALP expression under below-critical threshold BMP2 concentration conditions.

Moreover, we observed the trigger of the OPN expression, the other critical marker characteristic of osteoblastic differentiation dependent of Smad1 nuclear translocation (19), 17 h after the chemical or physical treatments in the presence of BMP2 for 60 min, whereas OPN did not yet express significantly within the same conditions without treatment. Therefore, these results indicate that the critical transcriptional steps of the BMP2-dependent osteoblastic terminal differentiation are considerably amplified, or can be triggered, by inhibition of the endocytosis of BMP2.

Finally, the repression of myogenin expression, a critical transcriptional event indicative of terminal myoblastic differentiation inhibition controlled by BMP2-dependent JunB expression (9), was found to be strongly amplified in response to BMP2 endocytosis inhibition after chemical and physical hyposmotic treatments. Thus this result indicates that one of the critical transcriptional steps of complete inhibition of myoblastic differentiation is also amplified by inhibition of BMP2 endocytosis.

Such results suggest that the inhibition of endocytotic internalization may prevent degradation of the BMP2-receptor complex in internal cytosolic compartments, thereby amplifying BMP2-dependent signal transduction, triggering rapid Smad1 translocation to the nucleus and resulting in the amplification of the Smad1-dependent transcriptional steps of the myoblast/osteoblast transdifferentiation. Alternatively, plasma membrane BMP2 receptor concentration may have been increased by possible exocytosis induced by the two treatments, amplifying the response of the cell to BMP2 (17). However, no change in plasma membrane BMP2 concentration was observed after either treatment at time 0 of the experiment, as verified by quantitative spectrofluorimetry. This therefore rules out this possibility, along with the possibility of a change in the affinity of BMP2 for its receptor caused by these two treatments, but there could also be a cooperative endocytosis-independent effect between the physical or chemical treatment and BMP2-dependent receptor activation at some point in the signal transduction pathway controlling the nuclear translocation of Smad1. However, the signal transduction pathway is direct in this case, with Smad1 phosphorylation the only signal required for the nuclear translocation of Smad1 (23). Therefore, the only possible cooperative effect would be the activation and translocation of Smad1 to the nucleus in response to both treatments in the absence of BMP2, which was ruled out experimentally. Moreover, the same putative cooperative signal transduction effect would be unlikely to occur in response to two
different physical and chemical treatments. Indeed, the common BMP2-specific effect of PEG-cyclodextrin and hyposmotic treatments leading rapid Smad1 nuclear translocation, and resulting in strong and more rapid JunB expression, is the inhibition of BMP2 endocytosis.

Endocytosis of ligand-activated receptors is already known to regulate the initiation of signaling events (3, 21). Here we show in C2C12 cells that the inhibition of endocytosis of BMP2 has a direct and highly sensitive role, amplifying Smad1-dependent critical transcriptional steps of the cell that control myoblast/osteoblast cell transdifferentiation. This effect is exerted from the translocation to the nucleus of the transcription factor Smad1, initiating transdifferentiation, to expression of the critical cell markers of the transdifferentiation themselves. Effectively, both the physical and chemical treatments inhibiting BMP2 endocytosis led to the amplification of the Smad1-dependent JunB expression increase characteristic of the initiation of the inhibition of myoblastic differentiation, to the amplification of the myogenin repression, indicative of terminal myoblastic differentiation inhibition, and to the Smad1-dependent ALP and OPN expression increase, characteristic of osteoblastic differentiation.

Interestingly, BMP2 endocytosis is modulated here by epigenetic factors instead of by genetic factors such as dynamic dominant mutations inhibiting clathrin-independent endocytosis (4). These results here thus show the direct influence of the ligand-receptor physical internalization regulated by endocytosis on the ligand-dependent cell differentiation process, independent of any putative genetic interaction between the mutated gene product and the signal transduction pathway.

Moreover, here endocytosis is modulated not only by chemical treatments but also by physical treatments generating osmotic forces exerted on the plasma membrane, mechanistically inhibiting budding vesiculation (16, 17). Importantly, BMP2 receptor-mediated endocytosis was thus mechanically inhibited by hyposmotic treatment in C2C12 cells, here inducing the amplification or trigger of the tested critical transcriptional steps involved in C2C12 myoblast/osteoblast transdifferentiation. This result therefore suggests that endocytosis mechanical modulation may act as a simple mechanical sensor, allowing the genome to sense a field of deformation resulting from mechanical constraints applied to the cell from the outside and to translate it into a genetic transcriptional signal. It has already been suggested that caveolae themselves act as direct mechanical sensors of mechanical strain on the plasma membrane (22). Here we suggest that there may be a specific cell differentiation process sensitive to mechanical deformation fields involving the modulation of endocytosis, in the presence of secreted signaling proteins only. This would make it possible for an organism to regulate cell differentiation as a function of cell tissue mechanical deformation in vivo, in the presence of morphogen-signaling molecules like BMP2 (1). Modulation of the endocytosis of signaling proteins under mechanical strain could therefore be a simple mechanotranscription translator, making it possible to modulate or to trigger cell differentiation and patterning in a tissue by mechanical deformation field gradients, for instance during embryogenesis. The morphogen responsible for tissue patterning would in this case be a mechanical deformation field, rather than a signaling protein concentration gradient. This property would lead to transcriptional patterning events, resulting specifically from interferences between biochemical patterning and a mechanical morphogenetic field of deformation patterning.

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