Initiation of Smad-Dependent and Smad-Independent Signaling via Distinct BMP-Receptor Complexes

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Background: BMP-2 (bone morphogenetic protein-2) signals via two types of transmembrane serine/threonine kinase receptors (BRI and BRII), which form heteromeric complexes prior to and after ligand binding. Within a BMP-bound receptor complex, BRII transphosphorylates and activates BRI-a for further signaling. We investigated which signaling pathway is initiated by BMP-2 via preformed receptor complexes versus BMP-2-induced signaling receptor complexes.

Methods: Immunofluorescence co-patching was used to study the oligomerization of receptors at the surface of live cells. Binding and chemical cross-linking of iodinated BMP-2 followed by immunoprecipitation was used to show association of receptors in the presence of ligand. Western blots with use of anti-phospho-Smad1 antibodies and reporter gene assays with use of SBE-lux were employed to show activation of the Smad pathway. Phosphorylation of p38-MAPK was shown by Western blots. Induction of alkaline phosphatase was determined by staining the cells. The cluster density of receptors was determined with use of image correlation spectroscopy.

Results and Conclusion: We showed that the Smad pathway is induced by preformed receptor complexes, whereas BMP-2-induced signaling complexes result in the activation of p38-MAPK. We also found evidence that the clustering of BRI-a at the membrane is altered in the presence of BRII, suggesting that it associates with existing clusters of BRII to initiate efficient Smad signaling. These data clearly demonstrate that it is critical to fully understand receptor oligomerization in order to estimate signaling outcome for distinct receptor and ligand mutants.

Clinical Relevance: The development of BMP-2 antagonists is of special importance for a number of human disorders caused by several members of the BMP/TGF-β (transforming growth factor-beta) superfamily. Since manipulation of BMP-signaling is complex, it is important to understand what influence it might have during the initiation of signaling—i.e., the oligomerization of BMP receptors to form a signaling receptor complex. There might be cases where either the Smad or the p38 pathway should be targeted.

BMP-2 (bone morphogenetic protein-2) signals via two types of transmembrane bound serine/threonine kinase receptors (BRI and BRII). Receptors of both types are needed to form a functional signaling complex, in which BRII transphosphorylates BRI at the GS box to activate its kinase. Activation of BRI induces phosphorylation of transiently associated Smad1, 5, or 8. These R-Smads translocate after oligomerization with Smad4 into the nucleus, where they regulate the transcription of specific target genes in concert with various DNA-binding proteins, co-activators, or co-repressor proteins[1].

In addition to the Smad-mediated canonical BMP signaling pathway, BMP also signals through other pathways. For example, BMP can activate several MAPKs, including extracellular signal-regulated kinases (ERKs) and p38 kinases[2-4]. Furthermore, BMP-2 promotes apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway[5].

Activation of p38 by BMP receptors requires TAK1, which is indirectly bound to BRI via bridging proteins XIAP and TAB1[6]. Studies of BMP and TGF-β (transforming growth factor-beta) receptors suggest that induction of the p38 pathway via TAK1 by these ligands is independent of the Smad proteins[5]. However it also has been shown for TGF-β-mediated p38 activation that only early responses (i.e., ten minutes) are mediated by TAK1, whereas late responses are mediated via GADD45β. The expression of GADD45β is induced by the TGF-β-mediated Smad pathway[7].

A crosstalk between Smads and the p38 pathway is further demonstrated by the inhibition via Smad6, which blocks both Smad1 activation by acting as a Smad4 decoy[8,9] and p38 activation through direct binding to TAK1[3].

It has been our interest to characterize the initiation of

Smad-dependent and Smad-independent BMP signaling at the level of its receptors. We therefore asked whether receptor oligomerization might determine the initiation of distinct signaling cascades. Here we show that the mode of BMP-receptor oligomerization determines whether Smads or p38 are directly activated. While binding of BMP-2 to preformed receptor complexes composed of BRII and BRI induces Smad activation, binding of BMP-2 to its high-affinity monomeric BRI induces first its homodimerization followed by BRII recruitment[10,11]. This complex (BISC [BMP-2-induced signaling complex]) results in a Smad-independent activation of p38[12].

Most recently, we have provided evidence that BRI-a-receptor clusters formed at the cell surface are rearranged when BRII is co-expressed, while BRII-receptor clusters do not change in the absence or presence of BRI. This suggests that BRII expression affects the organization of BRI in the membrane, pulling it into the clusters of BRII and enabling the formation of preformed receptor complexes at specific sites on the cell membrane, where efficient Smad signaling can occur.

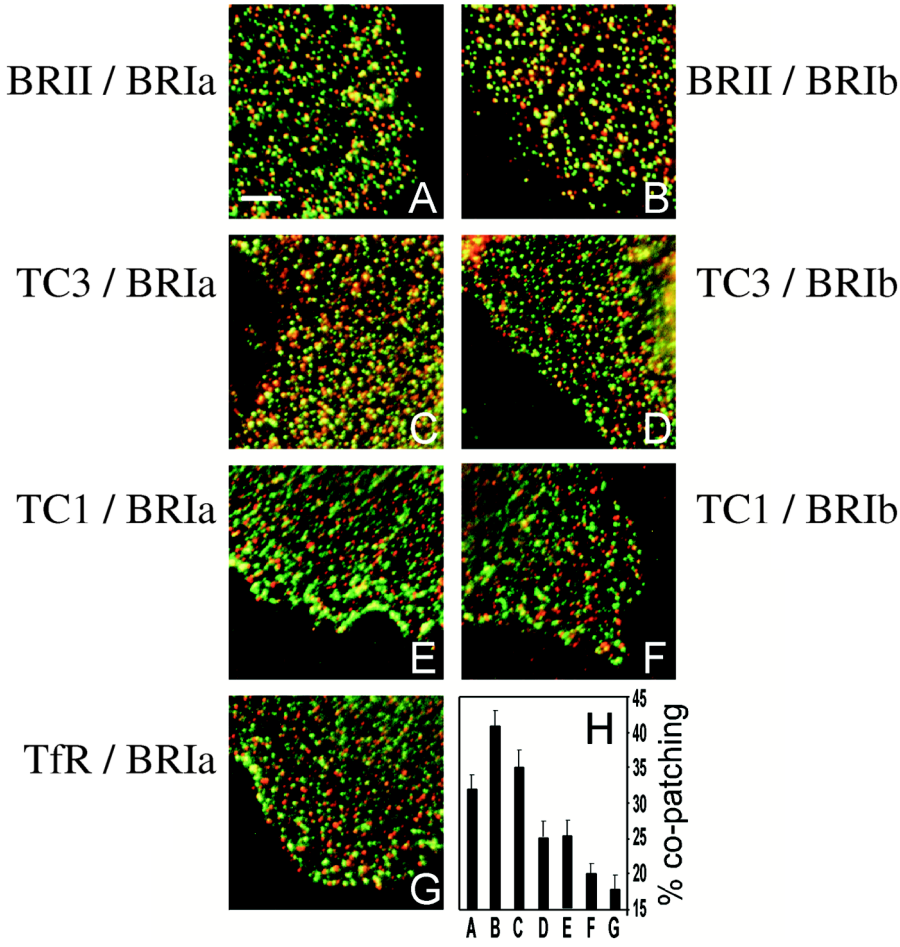

Fig. 1

BMP-receptor oligomerization in live cells. COS7 cells were transfected with various receptor combinations. One of the receptors was tagged with HA fusion protein (hemagglutinin protein), and the other was tagged (labeled) with myc protein. The receptors were labeled with antibodies recognizing the tag and fixed subsequently. Images were made and superimposed. Green (FITC, myc) or red (HA, Cy3) labeled receptors were detected as well as patches (yellow) in which both receptor subtypes were labeled. Bar = 20 μm. *A:* BRII-HA (red) and BRI-a-myc show a significant level of co-patching compared with the control (14% above the background level, $p < 0.001$ according to Student t test). *B:* Colocalization of BRI-b-HA with BRII-myc. The level of co-patching is 23% above background ($p < 0.001$). *C:* The level of co-patching of BRI-a-HA and BRII-TC3-myc is similar to that of BRII-HA with BRI-a-myc—i.e., 17% above background ($p < 0.001$). *D:* The colocalization of BRI-b-HA with BRII-TC3-HA is slightly above the background co-patching level (7%; $p < 0.001$). *E:* HA-BRI-a (red) and myc-TC1 (green) co-patching is only slightly above the background co-patching level (7%; $p < 0.001$). *F:* BRI-b-HA does not colocalize with BRII-TC1-myc; colocalization is 7% above background ($p > 0.1$). *G:* Control co-patching of two unrelated receptors, BRI-a-HA and the transferrin receptor. They show a low degree of copatching (18%), which resembles the background level when unrelated receptors are employed. *H:* Quantification of co-patching data from experiments A through G. (Reprinted, with permission, from: Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 pathways. J Biol Chem. 2002;277:5330-8.)

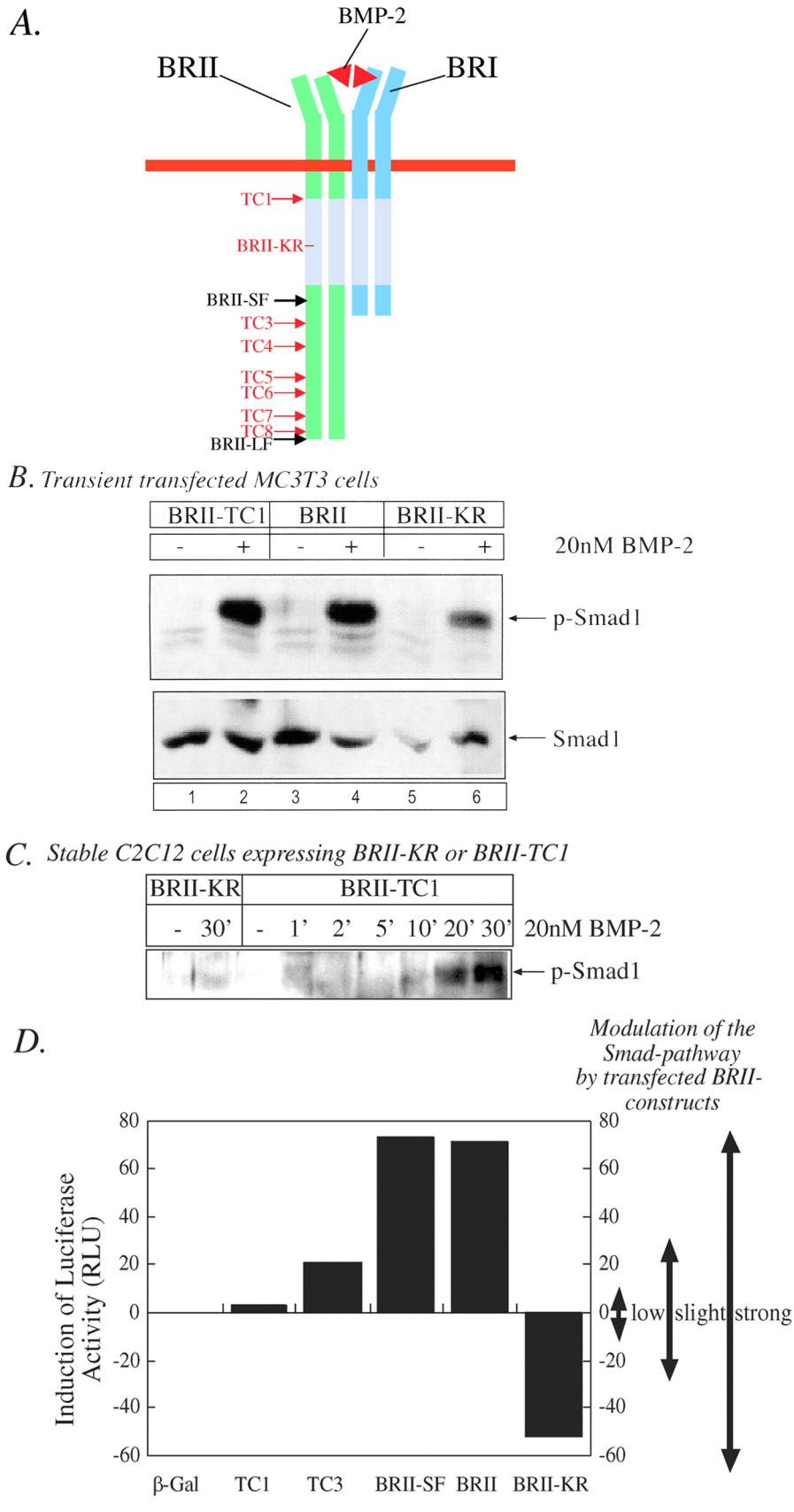

Fig. 2

BMP receptors and the Smad pathway. *A:* Schematic illustration of BRII mutants generated to determine receptor oligomerization. TC1 lacks nearly the entire cytoplasmic domain, TC3 stops directly after the kinase domain, and BRII-SF and BRII-LF are two naturally occurring splice variants of BRII. BRII-KR represents a kinase-deficient receptor due to a point mutation (K230R). *B:* Smad-phosphorylation in transiently transfected MC3T3 cells. After transfection, the cells were starved and then stimulated for thirty minutes with BMP-2. Equal amounts of cell lysate were subjected to Western blot analysis with use of antibodies against the C-terminal phosphorylated form of Smad1/5/8 (upper panel) or the non-phosphorylated form (lower panel). Whereas TC1 is not affecting BMP-2-induced Smad1/5/8 phosphorylation (lanes 1 and 2), BRII-KR acts in a dominant negative fashion compared with BRII (lanes 3 through 6). (Reprinted, with permission, from: Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 pathways. J Biol Chem. 2002;277:5330-8.) *C:* Using retroviral transduction, we generated C2C12 cell lines stably expressing indicated receptor variants. Examining the TC1 line, we showed that, with use of the same protocol as described above, TC1 cells showed Smad1/5/8 phosphorylation after ten minutes of ligand incubation, increasing after twenty minutes and with the strongest signal after thirty minutes. BRII-KR also acts in a dominant negative manner in the stable cell line. *D:* Influence of the BRII variants on the transcriptional activity of Smads. We transfected MC3T3 cells with the different receptor variants and pSBE and pRLTK and stimulated them for twenty-four hours with 10nM BMP-2. Here we show only the signals from the stimulated cells. "0" represents the induction by BMP-2 in mock transfected cells. TC1 has no influence on endogenous signaling, TC3 slightly induces Smad signaling, the naturally occurring variants BRII-SF and BRII strongly induce Smad signaling, and BRII-KR acts in a strongly dominant negative manner. (Reprinted, with permission, from: Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 pathways. J Biol Chem. 2002;277:5330-8.)

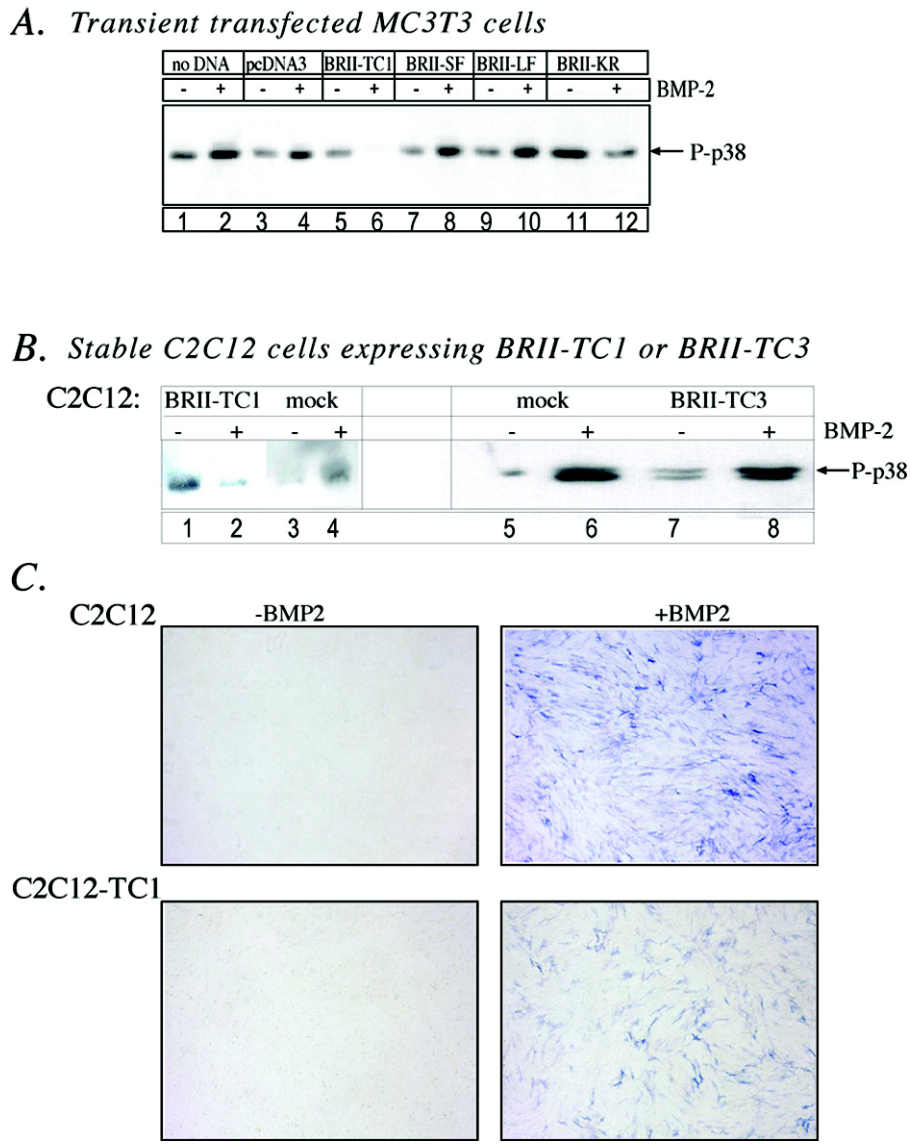

Fig. 3

BMP receptors and Smad-independent pathways. *A:* We transfected MC3T3 cells with the indicated BRII-receptor constructs and stimulated cells with 20nM BMP-2 for ninety minutes. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with use of an anti-phospho-p38 antibody (Promega, Madison, WI). The splice variants BRII-SF and BRII-LF behave like not-transfected or mock-transfected cells (lanes 1 through 4 and 7 through 10). BRII-TC1 and BRII-KR block p38 phosphorylation (lanes 5, 6, 11, and 12). (Reprinted, with permission, from: Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 pathways. J Biol Chem. 2002;277:5330-8.) *B:* Stable C2C12 cells were seeded on six-well dishes and stimulated for one hour with 20nM BMP-2. Cell lysates were analyzed by Western blotting with use of anti-phospho-p38 antibody. BRII-TC1 is blocking p38 phosphorylation in a dominant negative fashion (lanes 1 and 2), whereas the BRII-TC3 activates p38-like cells expressing endogenous wild-type receptors (lanes 5 through 8). This confirms the results obtained from transient transfections. *C:* C2C12 cells stably expressing BRII-TC1 and wild-type C2C12 cells were plated on six-well plates and stimulated with 50nM BMP-2 for three days. After fixing of the cells with formaldehyde, cells were stained for alkaline phosphatase and analyzed with a Nikon TE 2000U microscope (Nikon, Melville, NY), and images were made with a DXM1200 camera (Leica, Mannheim, Germany). In C2C12 cells, BMP-2 strongly induces alkaline phosphatase production. BRII-TC1-expressing cells show a reduction of alkaline phosphatase production in the presence of BMP-2.

Methods

Co-Patching

COS cells growing on glass coverslips were transfected with the different receptor constructs, which were tagged with different epitope tags (HA fusion protein [hemagglutinin protein] or myc protein). Forty-eight hours after transfection, the cells were blocked (goat IgG, 200 µg/mL, forty-five minutes; 4°C in Hank balanced salt solution with 20mM Hepes, pH 7.4, 1% BSA) and labeled in the cold with anti-HA (rabbit HA11, BabCO), anti-myc (mouse, BAbCO), or anti-B3/25 against the transferrin receptor (Roche Molecular Biochemicals, Indianapolis, IN) in the same buffer. Incubation with secondary antibodies (20 µg/mL each), IgG-Cy3-GαR (goat anti-rabbit) or FITC-GαM (goat anti-mouse; Jackson ImmunoResearch Labs, West Grove, PA), was for forty-five minutes at 4°C. After washing, the cells were fixed in methanol and acetone and were mounted before separate green and red fluorescence CCD (charge-coupled device) images were made. Superimposed red and green images were analyzed by counting the numbers of green, red, and yellow (superimposed red and green) patches, counting at least 100 patches per cell on ten to fifteen cells in each case. The percentage co-patching (percentage of a given tagged receptor in a patch with other receptors) is given by 100× (yellow/[yellow + red]) for the red-labeled receptors and 100× (yellow/[yellow + green]) for the green-labeled receptors[10]. Because these values were very close, a single value is depicted for each pair. The values shown are a mean and standard error of the mean of the percentage co-patching of the different receptor pairs.

Western Blots

For transient transfection, 1×10^5 MC3T3 cells were transiently transfected with different receptor constructs with use of lipofectamine (Gibco, Grand Island, NY). Twenty-four hours after

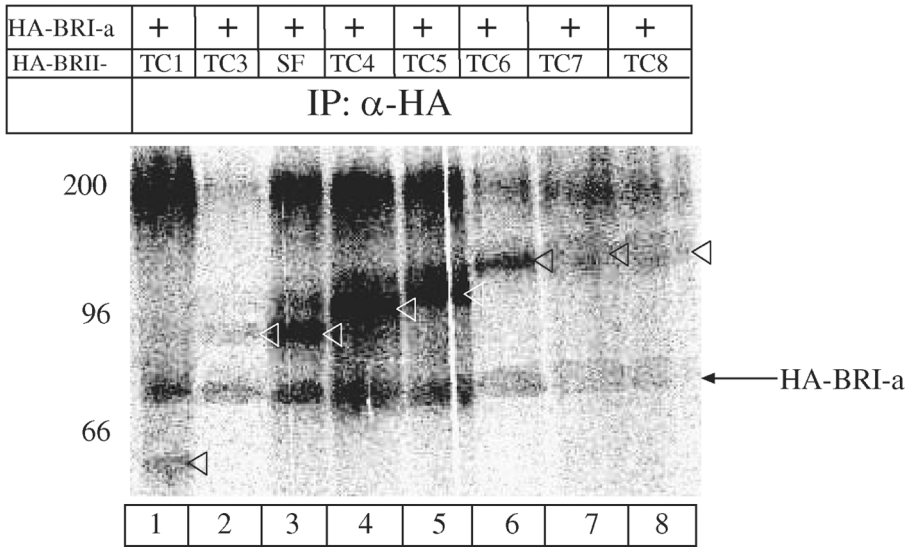

Fig. 4

Binding and chemical cross-linking of iodinated BMP-2 to BRII variants and BRI-a: COS7 cells were cotransfected with HA-tagged BRII truncations as well as HA-tagged BRI-a. After binding and cross-linking of 5nM ^{125}I-BMP-2, receptors were immunoprecipitated with anti-HA (12CA5) and protein A-Sepharose. Precipitates of one 100-mm dish were loaded on a sodium dodecyl sulfate gel and subjected to autoradiography. All BRII-TCs (marked by triangles) are expressed at the cell surface and form a complex with BRI-a-HA (black arrow) after ligand addition. (Reprinted, with permission, from: Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 pathways. J Biol Chem. 2002;277:5330-8.)

transfection (for Smads) or forty-four hours after transfection (for p38), cells were starved for twenty-four hours (for Smads) or five hours (for p38) in Dulbecco minimum essential medium supplemented with 0.5% fetal calf serum. Followed by incubation with 20nM BMP-2 for thirty minutes (Smads) or ninety minutes (p38). After that, cells were harvested and lysed in TNE buffer (see below), and the protein amount was determined (BCA assay). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

For stable lines, C2C12 cells were plated on a six-well dish (1×10^5). Forty-eight hours later, they were starved for twenty-four hours for Smad phosphorylation or three hours for p38 phosphorylation. After stimulation for thirty minutes for Smad phosphorylation or one hour for p38 phosphorylation with 20nM BMP-2, cells were lysed in 100 μL of TNE buffer (10mM Tris, pH 7.4; 150mM NaCl; 1% Triton X-100,

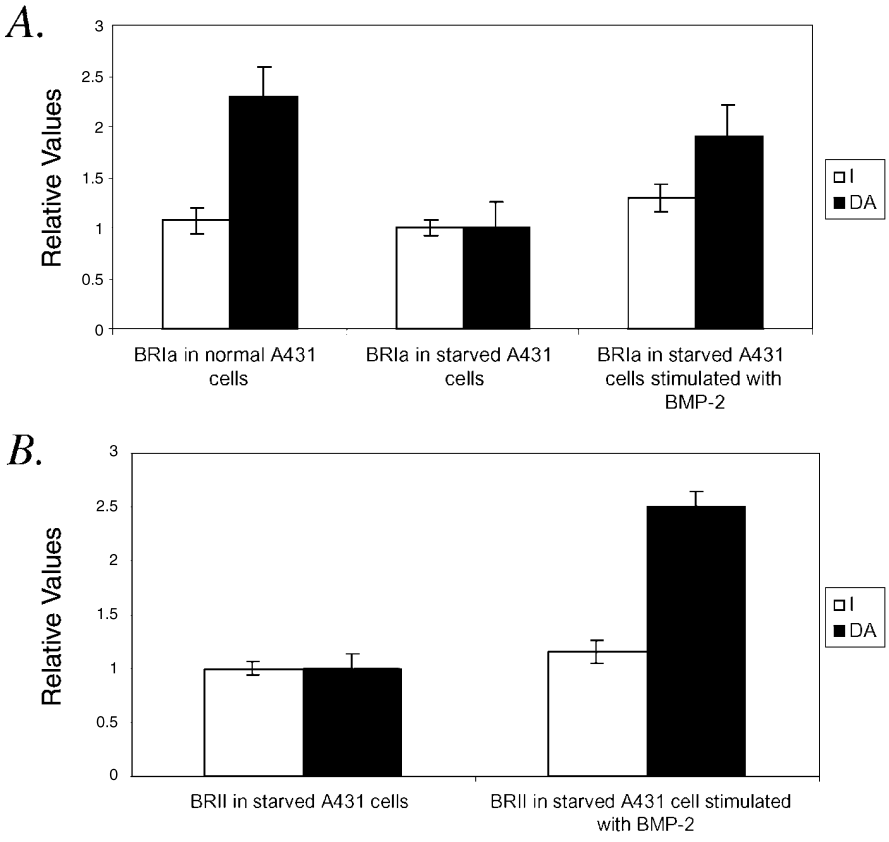

Fig. 5

Cluster density of BMP receptors in A431 cells. *A* and *B:* Relative degree of aggregation of the BMP receptors on the cell surface. Upon starvation of A431 cells, BRII is upregulated. This leads to a dispersion of BRI-a into smaller aggregates with less receptors in them. Stimulation of A431 cells with BMP-2 leads to a redistribution of BRI-a and BRII into larger clusters containing more receptors, as can be seen by an increase in the degree of aggregation. I = intensity and DA = degree of aggregation. As shown by Nohe et al.[24], there is no effect on BRII clustering when BRI-a is co-expressed.

and 1mM EDTA) with 1mM phenylmethylsulfonyl fluoride and protease-(Boehringer complete) as well as phosphatase inhibitors (50mM NaF, 10mM $Na_2P_2O_7$, and 25mM Na_3VO_4). After protein determination, equal amounts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Reporter Gene Assay

1×10^6 MC3T3 cells were electroporated with indicated receptor constructs and pSBE-luc[13] as a BMP-dependent reporter, as well as pRLTK for reference. After six hours in complete medium, the cells were starved with Dulbecco minimum essential medium with 0.5% fetal calf serum for five hours and stimulated for twenty-four hours with 10nM BMP-2. After cell lysis, luciferase activity was determined by using the dual luciferase system (Promega, Madison, WI).

Alkaline Phosphatase Stain

4.5×10^5 C2C12 cells were plated on a six-well dish, and after twenty-four hours they were starved in Dulbecco minimum essential medium with 0.5% fetal calf serum for five hours followed by ligand incubation (50nM BMP-2 in low-serum medium) for three days. The cells were then fixed with 3.7% formaldehyde and stained for alkaline phosphatase[14].

Binding and Cross-Linking

Transfected COS7 cells were incubated with 5nM ^{125}I BMP-2 in KRH with 0.5% fatty-acid-free BSA for two to six hours at 4°C. Iodination was done with use of the chloramine-T method[10]. Cross-linking was performed with dissuccinimidyl suberate and stopped by adding sucrose (ad 7%). After cell lysis (0.5% Triton X-100, 1mM EDTA in phosphate-buffered saline solution with phenylmethylsulfonyl fluoride and protease inhibitors), BRI-a was immunoprecipitated with use of 12CA5 monoclonal antibodies (20 µg/mL) and 30 µL protein A-Sepharose, and the immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cluster Density

A431 cells were grown in Dulbecco minimum essential medium on 22-mm coverslips with or without 10% fetal bovine serum. After seventy-two hours, cells were fixed with use of the acetone/methanol method and were labeled with use of a polyclonal antiserum against the BMP receptors followed by a rhodamine conjugated secondary antibody. Labeled cells were visualized with use of a BioRad confocal microscope (BioRad Laboratories, Hercules, CA), and image correlation spectroscopy calculations were performed as described elsewhere[15,16]. Applying image correlation spectroscopy, we can calculate the number of receptor clusters per unit area and the degree of aggregation. The degree of aggregation is the relative average number of proteins per cluster.

Results and Discussion

Using several complementary approaches (immunofluorescence co-patching and immunoprecipitations), we showed that BMP receptors reside in homomeric and heteromeric complexes at the cell surface prior to ligand binding. Heteromeric complexes composed of BRII and BRI-a or BRI-b are most prominent[10] (Fig. 1).

Preassembled receptors have been described for several receptors as the trimeric TNF (tumor necrosis factor) receptors[17], heteromeric IL-1 (interleukin-1) and IL-2 receptors[18], EGF (epidermal growth factor) receptor[19], and the homodimeric Epo-receptor[20]. Recently, for the TNF receptors, a PLAD (pre-ligand binding assembly domain) that is physically distinct from the ligand binding site was determined[17], while the Epo receptor uses the same site for receptor pre-assembly as it uses later for ligand binding[21,20].

In order to identify the sites essential for BRII and BRI preassembly, we generated a series of BRII truncation mutants (Fig. 2, *A*). We could show that the kinase domain of BRII is important for complex formation. Both alternative splice variants of BRII, resulting in a short form of the receptor (530aa) and a long form (1038aa), are able to form preformed receptor complexes. However, the deletion of the kinase domain (BRII-TC1) results in complete loss of receptor preassembly[12] (Fig. 1). This shows that domains important for receptor preassembly are localized within the kinase domain of BRII.

Since BRII-TC1 lacks the kinase domain, it has the potential to act as a dominant negative receptor; however, a prerequi-

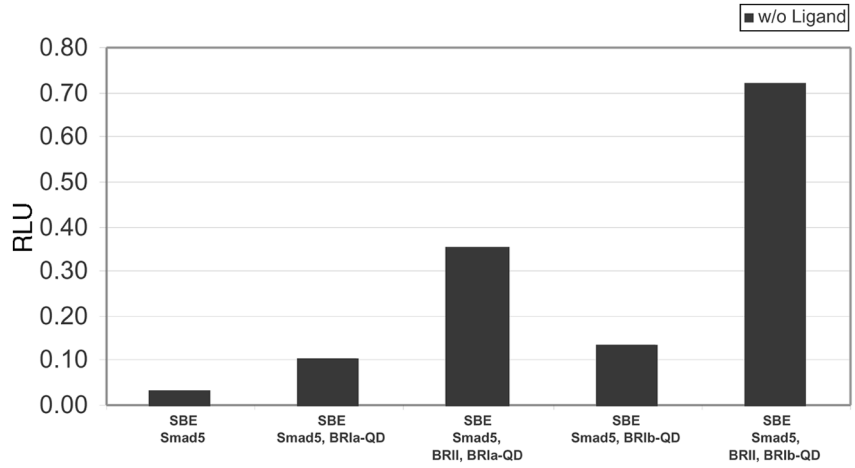

Fig. 6

BRII co-expression enhances ligand independent signaling of constitutive active BRI. MC3T3 cells were cotransfected with pRL-TK, pSBE-luc, Smad5, and indicated receptor constructs. Following starvation (twenty-nine hours), cell lysates were prepared and luciferase activity was measured. Data represent one of three independent experiments. The constitutive active BRI receptors signal without ligand addition. This, however, is increased when BRII is co-expressed. (RLU = relative light units.)

site for a dominant activity is that BRII-TC1 has the same oligomerization behavior as the wild-type receptor. Since BRII-TC1 is not present in preformed receptor complexes, as shown in Figure 1, BRII-TC1 is not able to influence signaling initiated from these heteromeric complexes after BMP-2 stimulation. Measurements of the BMP-2-induced Smad pathway by the use of an anti-phospho-Smad1-specific antibody[22,23] (Fig. 2, *B* and *C*) as well as reporter gene assays with use of the SBE-lux (Smad-binding-element[13]) revealed that expression of BRII-TC1 in BMP-responsive cells did not alter the Smad induction in a dominant fashion (Fig. 2, *D*). This was shown in transiently transfected MC3T3 cells (Fig. 2, *B*) as well as in C2C12 cells, which stably express BRII-TC1 after retroviral transduction (Fig. 2, *C*). However, in the same cells, BMP-2-induced p38 activation (Fig. 3, *A* and *B*) and the induction of alkaline phosphatase (Fig. 3, *C*) were reduced in a dominant negative fashion[12]. Since we showed that BRII-TC1 is still able to oligomerize with BRI after addition of BMP-2, it can be recruited into BMP-2-induced signaling complexes (Fig. 4). This suggests that the kinase (plus tail) domain of BRII is not essential for complex formation resulting in p38 induction. However, the kinase activity of BRII is important for p38 activation. Furthermore, it suggests that p38 is an essential upstream component of alkaline phosphatase induction by BMP-2.

To provide further evidence for our model, we used the BRII-SF mutant, BRII-KR, which resembles the short form of BRII carrying an inactive kinase as a result of the point mutation K230R. This receptor behaves as the wild-type receptor with respect to its oligomerization mode. However, because of its kinase deficiency, BRII-KR has a dominant negative effect on both the Smad pathway and the p38 pathway. This suggests that the kinase domain is essential for preformed receptor complex formation and its activity is required for both Smad and p38 activation.

Next we analyzed the formation of receptor clusters at the cell surface with image correlation spectroscopy. Image correlation spectroscopy involves the calculation of the two-dimensional autocorrelation function of the spatial fluctuations in intensity across a high-magnification image[15,16]. We showed that BRI-a-receptor clusters are rearranged when BRII

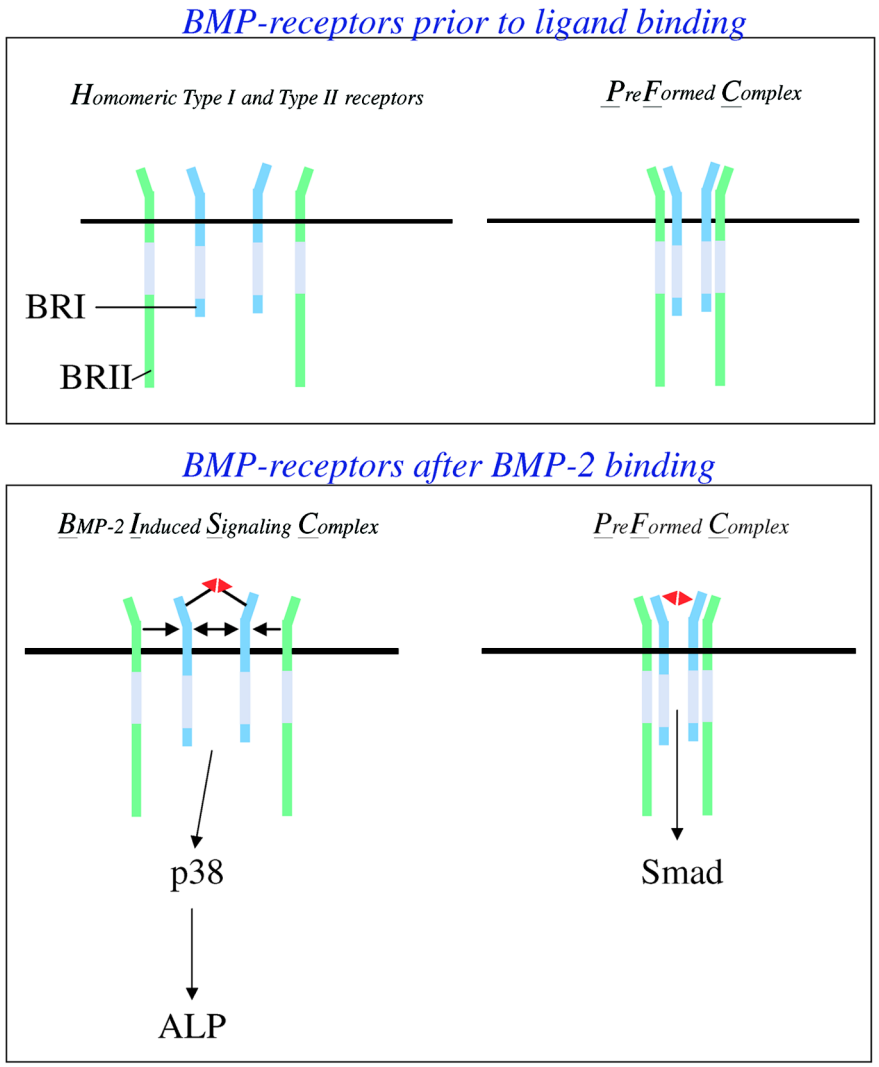

Fig. 7

Initiation of Smad-dependent and Smad-independent signaling from the plasma membrane. Before ligand binding, BMP receptors exist at the cell surface as homomeric type-I and type-II receptors or as preformed complexes composed of both types of receptors. BMP-2 has at least two options to activate signaling: either it binds to its high-affinity BRI receptor, which causes recruitment of BRII into a heteromeric complex (BMP-2-induced signaling complex), or it binds to preformed receptor complexes. Activation of BMP-2-induced signaling complex leads to initiation of the p38 pathway, which is Smad-independent, while activation of preformed receptor complexes leads to initiation of the Smad pathway. The site where preformed receptor complexes are formed at the cell surface might determine the site to activate Smad signaling. This is determined by the BRII receptor.

is co-expressed, while BRII-receptor clusters do not change in the presence of BRI[24]. This demonstrates that BRI organization in the membrane is altered in the presence of BRII, reflecting its association with BRII clusters (which are not modified). These specific clusters on the cell membrane reflect the sites of efficient Smad signaling[24] (Fig. 5). This is also seen for the constitutive active mutant of BRI-a. BRI-a-Q233D was described as activating the Smad pathway independent of ligand[25]. However, as we showed, cotransfection of BRII further enhances signaling (Fig. 6). This may be due to formation of heterooligomeric receptor complexes, the preformed receptor complexes of BRI-a-Q233D, and BRII at sites where efficient Smad signaling can occur[24]. The localization of BRII at specific domains on the cell surface seems to represent the sites where Smad signaling is initiated (Fig. 7).

From these studies, we concluded that BMP-receptor mutants such as truncations (TC1) or point-mutations (KR, kinase-dead, or Q233D, constitutively active) need to be analyzed for their oligomerization potential to fully understand their signaling impact. Since the oligomerization potential of BMP receptors is more flexible than that of the TGF-β receptors, it is critical to assume that BMP-receptor mutants analogous to the TGF-β receptors result in the same signaling outcome. Results with BRII-truncations and constitutive active BRI clearly demonstrate differences between BMP and TGF-β receptors. ■

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References

1. **Miyazono K.** TGF-beta signaling by Smad proteins. *Cytokine Growth Factor Rev.* 2000;11:15-22.
2. **Iwasaki S, Iguchi M, Watanabe K, Hoshino R, Tsujimoto M, Kohno M.** Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. *J Biol Chem.* 1999;274:26503-10.
3. **Kimura N, Matsuo R, Shibuya H, Nakashima K, Taga T.** BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. *J Biol Chem.* 2000;275:17647-52.
4. **Kozawa O, Hatakeyama D, Uematsu T.** Divergent regulation by p44/p42 MAP kinase and p38 MAP kinase of bone morphogenetic protein-4-stimulated osteocalcin synthesis in osteoblasts. *J Cell Biochem.* 2002;84:583-9.
5. **Hay E, Lemonnier J, Fromigue O, Marie PJ.** Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. *J Biol Chem.* 2001;276:29028-36.
6. **Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K.** XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J.* 1999;18:179-87.
7. **Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K, Saito H.** Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *EMBO J.* 2002;21:6473-82.
8. **Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K.** Smad6 inhibits signalling by the TGF-beta superfamily. *Nature.* 1997;389:622-6.
9. **Hata A, Lagna G, Massague J, Hemmati-Brivanlou A.** Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* 1998;12:186-97.
10. **Gilboa L, Nohe A, Geissendorfer T, Sebald W, Henis YI, Knaus P.** Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors. *Mol Biol Cell.* 2000;11:1023-35.
11. **Knaus P, Sebald W.** Cooperativity of binding epitopes and receptor chains in the BMP/TGFbeta superfamily. *Biol Chem.* 2001;382:1189-95.
12. **Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P.** The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J Biol Chem.* 2002;277:5330-8.
13. **Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W.** Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. *J Biol Chem.* 1998;273:21145-52.
14. **Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T.** Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol.* 1994;127:1755-66.
15. **Nohe A, Petersen NO.** Image correlation spectroscopy: exploring intramolecular interactions. *Biophotonics Internat.* 2002;9:39-52.
16. **Petersen NO.** Fluorescence correlation spectroscopy. In: Rigler R, Elson ES. *Fluorescence correlation spectroscopy: theory and applications.* New York: Springer; 2001.
17. **Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ.** A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science.* 2000;288:2351-4.
18. **Damjanovich S, Bene L, Matko J, Alileche A, Goldman CK, Sharrow S, Waldmann TA**Preassembly of interleukin 2 (IL-2) receptor subunits on resting Kit 225 K6 T cells and their modulation by IL-2, IL-7, and IL-15: a fluorescence resonance energy transfer study*Proc Natl Acad Sci USA*1997;94:13134-9.
19. **Gadella TW Jr, Jovin TM.** Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J Cell Biol.* 1995;129:1543-58.
20. **Remy I, Wilson IA, Michnick SW.** Erythropoietin receptor activation by a ligand-induced conformation change. *Science.* 1999;283:990-3.
21. **Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson IA.** Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science.* 1999;283:987-90.
22. **Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engstrom U, Heldin CH, Funa K, ten Dijke P.** The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* 1998;434:83-7.
23. **Ishisaki A, Yamato K, Hashimoto S, Nakao A, Tamaki K, Nonaka K, ten Dijke P, Sugino H, Nishihara T.** Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. *J Biol Chem.* 1999;274:13637-42.
24. **Nohe A, Keating E, Underhill TM, Knaus P, Petersen NO.** Effect of the distribution and clustering of BMP receptors on the activation of signaling pathways. *J Cell Sci.* In press.
25. **Akiyama S, Katagiri T, Namiki M, Yamaji N, Yamamoto N, Miyama K, Shibuya H, Ueno N, Wozney JM, Suda T.** Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. *Exp Cell Res.* 1997;235:362-9.