Menin Is Required for Bone Morphogenetic Protein 2- and Transforming Growth Factor β-regulated Osteoblastic Differentiation through Interaction with Smads and Runx2

Hideaki Sowa‡, Hiroshi Kaji†, Geoffrey N. Hendy§, Lucie Canaff§, Toshihisa Komori¶, Toshitsugu Sugimoto‡**, and Kazuo Chihara‡

From the ‡Division of Endocrinology/Metabolism, Neurology and Hematology/Oncology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe 6500017, Japan, the §Departments of Medicine, Physiology and Human Genetics, McGill University, Montreal, Quebec H3A 1A1, Canada, and the ¶Department of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka 565, Japan

Menin, the product of the multiple endocrine neoplasia type 1 (MEN1) gene, is required for commitment of multipotential mesenchymal stem cells to the osteoblast lineage, however, it inhibits their later differentiation (Sowa, H., Kaji, H., Canaff, L., Hendy, G.N., Tsukamoto, T., Yamaguchi, T., Miyazono, K., Sugimoto, T., and Chihara, K. (2003) J. Biol. Chem. 278, 21058–21069). Here, we have examined the mechanism of action of menin in regulating osteoblast differentiation using the mouse bone marrow stromal ST2 and osteoblast MC3T3-E1 cell lines. In ST2 cells, reduced menin expression achieved by transfection of menin antisense DNA (AS) antagonized bone morphogenetic protein (BMP)-2-induced alkaline phosphatase activity and osteocalcin and Runx2 mRNA expression. Menin was co-immunoprecipitated with Smad1/5 in ST2 and MC3T3-E1 cells, and inactivation of menin antagonized BMP-2-induced transcriptional activity of Smad1/5 in ST2 cells, but not MC3T3-E1 cells. Menin was co-immunoprecipitated with the key osteoblast regulator, Runx2, and AS antagonized Runx2 transcriptional activity and the ability of Runx2 to stimulate alkaline phosphatase activity only in ST2 cells but not in MC3T3-E1 cells. In the osteoblast MC3T3-E1 cells, transforming growth factor-β and its signaling molecule, Smad3, negatively regulated Runx2 transcriptional activity. Menin and Smad3 were co-immunoprecipitated, and combined menin and Smad3 overexpression antagonized, whereas menin and the dominant-negative Smad3C together enhanced BMP-2-induced transcriptional activity of Smad1/5 and Runx2. Smad3 alone had no effect. Therefore, menin interacts physically and functionally with Runx2 in uncommitted mesenchymal stem cells, but not in well differentiated osteoblasts. In osteoblasts the interaction of menin and the transforming growth factor-β/Smad3 pathway negatively regulates the BMP-2/Smad1/5- and Runx2-induced transcriptional activities leading to inhibition of late-stage differentiation.

Bone modeling and remodeling are essential for development, maturation, maintenance, and repair of bones. During bone modeling and remodeling, multipotential mesenchymal stem cells, derived from mesoderm, become osteoprogenitors and commit to the osteoblast lineage. Theretore, the cells undergo further differentiation, exhibiting osteoblast-specific phenotypes, such as the expression of type I collagen, alkaline phosphatase (ALP), and osteocalcin (OCN). Along with this maturation process of osteoblasts, extracellular matrix is mineralized or calcified and bone is formed. These sequential events are regulated by several hormones, growth factors, and cytokines. The intracellular signaling pathways triggered by these factors cross-talk to each other, forming a complex network system. The output of these signaling pathways drives the transcriptional factors, such as Runx2 (1–8), which are important for osteoblast differentiation.

Multiple endocrine neoplasia type 1 (MEN1, Mendelian Inheritance in Man (MIM) 131100) is a cancer predisposition syndrome inherited as a dominant trait. It affects a variety of endocrine tissues, in particular parathyroids, endocrine pancreas, and anterior pituitary. The MEN1 gene located on chromosome 11q13 (9) has been identified (10, 11) and of the more than 300 independent germline and somatic mutations scattered throughout the protein coding region (12), many are clearly inactivating, given rise to a truncated product. Consequently, lack of both MEN1 gene alleles leads to tumor development consistent with the 610-amino acid protein product, menin, acting as a tumor suppressor. Mice with heterozygous menin inactivation exhibit a phenotype similar to that of the human disorder and endocrine tumors develop later in life (13, 14). Menin is predominantly located in the nucleus (15, 16). However, the physiological functions of menin are unclear, but may be related to transcriptional regulation (15), cell cycle control (16), and interactions with various proteins including transcription factors have been demonstrated (17–24). Menin is widely expressed from an early developmental stage and found in both nonendocrine...
and endocrine tissues (25, 26). Recently, Crabtree et al. (14) reported that homozygous menin inactivation in mice was embryonic lethal and some fetuses exhibited clear defects in cranial and facial development. Because cranial bones are formed by intramembranous ossification, these findings suggested that menin might play an important role in the commitment of multipotential mesenchymal stem cells to the osteoblast lineage and osteoblast differentiation.

Transforming growth factor (TGF-β) superfamily members play a central role in a broad range of cellular responses including growth and differentiation. Within this superfamily, bone morphogenetic proteins (BMPs) and TGF-β are critical regulators of bone formation. BMPs are originally identified as molecules that induce ectopic bone and cartilage formation in the rat (27–29). BMPs induce the commitment of mesenchymal cells into osteochondrocyte progenitors and regulate growth and differentiation of these osteoblast and chondrocyte lineage cells (30–32). TGF-β is most abundant in bone matrix (33) where it is stored in an inactive form and upon release, it is activated in the bone microenvironment (34). Whereas TGF-β may provide competence for early stage osteoblastic differentiation, it inhibits late-stage osteoblast differentiation (35).

BMPs and TGF-β act through a cell-surface complex of type I and type II transmembrane serine/threonine kinase receptors. In the presence of ligand, the receptors associate and the type II receptor phosphorylates the type I receptor, which propagates a signal by phosphorylating receptor-regulated Smads (R-Smads) at their carboxyl termini and activating them (36, 37). BMP receptors activate Smad1, -5, and -8, and TGF-β receptors activate Smad2 and -3. Activated Smads form stable complexes with the common Smad (C-Smad), Smad4, and the complex translocates to the nucleus and regulates transcriptional responses initiated by BMP or TGF-β.

Runx2, also called Cbfa1, a runt family transcription factor, plays a central role in osteoblast differentiation (2–5). Targeted disruption of Runx2 results in the complete loss of functional osteoblasts indicating that Runx2 is essential for bone formation (2–5). Haploinsufficiency of the Runx2 gene in humans leads to cleidocranial dysplasia with delayed closure of sutures, small or absent clavicles, tooth anomalies, and short stature. Runx2−/− mice show a similar phenotype characterized by abnormal ossification suggesting that the expression level of Runx2 is critical for proper ossification (4, 5). Runx2 is a common target of BMP-2 and TGF-β, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the mesenchymal C2C12 cell line (38).

Our recent study (22) suggested that menin promotes the commitment of multipotential mesenchymal stem cells into the osteoblast lineage and inhibits their later differentiation. However, the molecular mechanisms underlying the actions of menin in this regard are not known. In the present study, we have examined the interactions of menin with BMP-2 and TGF-β signaling molecules, the Smads, and the pivotal transcriptional regulator of osteogenesis, Runx2, in both a multipotential mesenchymal cell line and an osteoblast cell line.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant BMP-2 was kindly provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Human recombinant TGF-β1, anti-myc mouse monoclonal antibody, and anti-FLAG mouse monoclonal antibody were from Sigma (St. Louis, MO). The menin rabbit polyclonal antibody was raised against a decapeptide (synthesized by solid-phase chemistry at the Peptide Synthesis Facility of the Sheldon Biotechnology Centre of McGill University) corresponding to amino acids 476–489 of menin (a sequence that is completely conserved between human and mouse) with an additional cysteine residue at the carboxyl terminus. The peptide was coupled via the cysteine residue to keyhole limpet hemocyanin. The antiserum was immunoaffinity purified before use, and, by Western blot of a variety of cell lines of different species, shown to recognize human and rodent menin with similar affinity (16).

Cell Culture—Mouse bone marrow ST2 and osteoblastic MC3T3-E1 cell lines were cultured in α-minimal essential medium (containing 50 µg/ml ascorbic acid) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). The medium was changed twice a week.

Construction of Expression Plasmids and Stable Transfection—For the antisense construct, human menin cDNA was cloned in an antisense orientation into the EcoRI site of pcDNA 3.1 (+). Antisense menin cDNA (AS) or empty vector (V) (each 3 µg) were transfected into ST2 cells (Invitrogen). Six hours after transfection, the cells were fed with fresh α-minimal essential medium containing 10% FBS. Reduced expression of menin by AS was detected by immunoblot analysis using the polyclonal anti-menin antibody. To rule out the possibility of clonal variation, we characterized at least three independent clones for each transfection. V-transfected cells were used as the control.

Construction of Expression Plasmids and Transient Transfection—The human menin cDNA, the myc-tagged Smad3 and FLAG-tagged Smad1/Smad5 vectors were prepared as previously described (22). A mutant form of myc-tagged Smad3 (Smad3AC), in which the MH2 domain corresponding to amino acid residues 278–425 was removed (22), was kindly provided by Dr. Y. Chen. These constructs or empty vector (V) (each 3 µg) were transfected into ST2 and MC3T3-E1 cells with LipofectAMINE (Invitrogen). Six hours later, the cells were fed with fresh medium containing 10% FBS. Forty-eight hours later, the transfected cells were harvested for the experiments.

Luciferase Assay—Cells were seeded at a density of 2 × 10⁵ per 6-well plate. Twenty-four hours later, cells were transfected with 3 µg of the reporter plasmid (3GC2 lux, OSC-luc or 3TP-Lux) with the pCH110 plasmid expressing β-galactosidase (1 µg) in each well, using Lipo-

Reference.
Menin Inactivation Antagonizes BMP-2-induced Osteoblast Differentiation in ST2 Cells—In our previous study, menin inactivation with menin antisense oligonucleotides antagonized BMP-2-induced osteoblast differentiation in the mouse mesenchymal C3H10T1/2 (10T½) cell line (22). In the present study, menin was inactivated with the transfection of AS menin DNA in a different mouse mesenchymal cell line, ST2, to confirm the effects of menin inactivation in this type of stem cell. The endogenous expression of menin was markedly reduced in AS-transfected ST2 cells (Fig. 1A), suggesting that AS is a useful tool for menin inactivation. AS antagonized BMP-2-induced ALP activity as well as the expression of Runx2 and OCN mRNA in these cells (Fig. 1, B and C), consistent with our findings in 10T½ cells (22). These results indicate that menin inactivation with AS antagonizes BMP-2-induced osteoblast differentiation of mesenchymal cells.

Menin Physically and Functionally Interacts with Smad1/5 in ST2 Cells—Smad1 and Smad5 are crucial mediators in BMP signaling pathways. We first examined the interaction of menin with Smad1/5 in ST2 cells. In these cells co-transfected with menin and Smad5, the two proteins were co-immunoprecipitated (Fig. 2A), indicating that menin physically interacts with Smad5. These data were consistent with our previous findings obtained in COS-7 cells (22). Moreover, this co-immunoprecipitation between menin and Smad5 was enhanced in the nucleus when the cells were stimulated with additional BMP-2 (Fig. 2B). However, co-immunoprecipitation was not observed in the presence and absence of BMP-2, when cytoplasmic extracts were employed (data not shown). Furthermore, we examined the effects of menin inactivation with AS on the transcriptional activity of Smad5, employing 3GC2-lux, which has Smad1/5 responsive elements driving a luciferase reporter gene (22). AS inhibited the transcriptional activity of Smad5 in ST2 cells (Fig. 2C). Similar data were obtained with Smad1 (data not shown). These findings indicate that menin physically and functionally interacts with Smad1/5 in ST2 cells.

Menin Physically and Functionally Interacts with Runx2 in ST2 Cells—Runx2 is a key target of the BMP-2 signaling pathway in osteoblast differentiation and the BMP-2/Runx2 cascade is important for the commitment of mesenchymal stem cells into the osteoblast lineage (38). We therefore investigated whether menin interacts with Runx2 in ST2 cells. After menin and Runx2 were co-transfected in ST2 cells they were co-immunoprecipitated (Fig. 3A), indicating that menin physically interacts with Runx2 in mesenchymal cells. Moreover, the co-immunoprecipitation between menin and Runx2 was enhanced in the nucleus when the cells were stimulated with additional BMP-2 (Fig. 3B). However, co-immunoprecipitation was not observed in the presence and absence of BMP-2, when cytoplasmic extracts were employed (data not shown). We next examined the effects of menin inactivation on the transcriptional activity of Runx2, using the OSC-luc reporter construct that has a Runx2-responsive element (42). The overexpression of Runx2 increased the activity of the luciferase reporter (Fig.
When AS was co-transfected with Runx2 in these cells, the luciferase activity was significantly reduced (Fig. 3C). These findings indicate that inactivation of menin antagonized the transcriptional activity of Runx2 in mesenchymal cells. It is possible that the reduction of Runx2 activity with menin inactivation reduces osteoblast differentiation markers such as ALP activity and OCN expression in ST2 cells. We therefore performed ALP activity assay in Runx2- and/or AS-transfected ST2 cells. Because Runx2 alone only modestly increased ALP activity and stimulated OCN expression in ST2 cells (data not shown), we examined the effects of menin inactivation on Runx2-induced ALP activity in the presence of BMP-2. As shown in Fig. 3C, Runx2 enhanced ALP activity induced by BMP-2. AS significantly antagonized BMP-2-induced ALP activity in V-transfected cells (Figs. 1B and 3D). Moreover, AS antagonized Runx2-induced enhancement of ALP activity (Fig. 3D). The data of Fig. 3D indicate there are Runx2-dependent and -independent components to the BMP-2 stimulation of ALP activity. The Runx2-dependent component was abrogated by menin inactivation, whereas the Runx2-independent pathway was not so affected. These data suggest that menin inactivation antagonizes osteoblast differentiation induced by Runx2.

Menin Inactivation Does Not Affect the Nuclear Accumulation of Smad1/5 and Runx2 in ST2 Cells—We examined the mechanism by which menin inactivation inhibits the transcriptional activity of Smad1/5 and Runx2 in multipotential mesenchymal stem cells. Because the nuclear accumulation of Smad1/5 and Runx2 are essential for their actions as transcriptional factors, we first investigated their levels in nuclear extracts of ST2 cells. Menin inactivation with AS did not affect the BMP-2-induced nuclear accumulation of Smad1, Smad5, or Runx2 in ST2 cells (Fig. 4). Immunoblotting with TF11H89 was used as the nuclear fraction protein control (16). These results...
suggest that menin inactivation inhibits the transcriptional activity of Smad1/5 and Runx2 after their translocation into nucleus.

Examination of Interaction of Menin with Smad1/5 and Runx2 in MC3T3-E1 Cells—Menin inactivation promoted osteoblast differentiation in MC3T3-E1 cells, which have already committed to the osteoblast lineage, while it antagonized osteoblast differentiation in uncommitted mesenchymal cells (22). These findings suggest that the interactions of menin with the BMP-2 signaling pathway and Runx2 might be different depending upon the osteoblast differentiation stage. We therefore examined the interactions of menin with Smad1/5 and Runx2 in MC3T3-E1 cells. Menin was co-immunoprecipitated with Smad1/5 in MC3T3-E1 cells (Fig. 5A) and this co-immunoprecipitation was enhanced in the nucleus when the cells were stimulated with BMP-2 (Fig. 5B). However, the co-immunoprecipitation was not observed in the presence and absence of BMP-2, when cytoplasmic extracts were employed (data not shown). However, AS did not affect the Smad5-induced luciferase activity of 3GC2-lux (Fig. 5C). Similar data were obtained for Smad1 (not shown). These findings suggest that menin interacts with Smad1/5 physically but not functionally, in well differentiated osteoblasts. Moreover, menin was not co-immunoprecipitated with Runx2, either in whole cell extracts or in nuclear extracts of cells stimulated (or not) with BMP-2 (Fig. 6, A and B), and AS did not affect the Runx2-induced luciferase activity of the OSC-luci gene in MC3T3-E1 cells (Fig. 6C). These findings indicate that menin interacts neither physically nor functionally with Runx2 in well differentiated osteoblasts.

TGF-β and Smad3 Negatively Regulate Runx2 Transcriptional Activity in MC3T3-E1 Cells—We hypothesized that some signaling pathways, for example, for TGF-β that has been reported to inhibit BMP-induced osteoblast differentiation (35), might negatively regulate the BMP-2-Runx2 cascade in MC3T3-E1 cells. TGF-β, as well as Smad3, suppressed the Runx2-driven luciferase activity of the OSC-luci construct in MC3T3-E1 cells (Fig. 7, A and B).

Interaction of Menin and Smad3 in MC3T3-E1 Cells—The above findings raised the possibility that menin and Smad3 may interact to negatively regulate the BMP-2-induced effects of Runx2 in later stage osteoblasts. First, we examined the interaction of menin and Smad3 in MC3T3-E1 cells. Menin was co-immunoprecipitated with Smad3 (Fig. 8A). Moreover, the co-immunoprecipitation was enhanced with TGF-β in the nucleus (Fig. 8B). However, the co-immunoprecipitation was not observed in the presence and absence of TGF-β, when cytoplasmic extracts were employed (data not shown). Furthermore, AS antagonized the Smad3-driven lu-
Experimental Procedures."

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**Fig. 6. Interaction of menin and Runx2 in MC3T3-E1 cells.** A, menin was transfected into MC3T3-E1 cells with Runx2. Forty-eight h later, cell extracts were immunoprecipitated (IP) with anti-Runx2, followed by immunoblotting (IB) with anti-menin, as described under "Experimental Procedures." B, menin was transfected into MC3T3-E1 cells with Runx2. Forty-eight h later, cells were treated with BMP-2 (100 ng/ml) for 2 h. Then, nuclear extracts were made and immunoprecipitated (IP) with anti-Runx2, followed by immunoblotting (IB) with anti-menin, as described under "Experimental Procedures." C, the Runx2 reporter construct, OSC-luci, was transfected into cells together with V or AS, and Runx2. Twenty-four h later relative luciferase activity was measured as described under "Experimental Procedures." Each value is the mean ± S.E. of 4 determinations.

**Fig. 7. Effects of TGF-β and Smad3 on transcriptional activity of Runx2 in MC3T3-E1 cells.** A, the Runx2 reporter construct, OSC-luci, was transfected into MC3T3-E1 cells with V or Runx2, and the cells were treated without or with TGF-β (5 ng/ml) for 24 h. Relative luciferase activity was measured as described under "Experimental Procedures." Each value is the mean ± S.E. of 4 determinations. *, p < 0.01, compared with the corresponding TGF-β-untreated group. B, OSC-luci was transfected into MC3T3-E1 cells together with V or Runx2, and/or Smad3. Twenty-four h later, relative luciferase activity was measured in cell extracts as described under "Experimental Procedures." Each value is the mean ± S.E. of 4 determinations. *, p < 0.01, compared with the corresponding V-transfected group.

Categorization activity of the Smad3-response element-containing 3TP-lux reporter construct (Fig. 8C). These data indicate that menin physically and functionally interacts with Smad3 in well differentiated osteoblasts.

**Effects of Menin and Smad3 on Smad5 and Runx2 Transcriptional Activity in MC3T3-E1 Cells—**We next examined the consequences of the interaction between menin and Smad3 on the transcriptional activity of Smad5 and Runx2 in MC3T3-E1 cells. Smad3 and menin overexpression antagonized the BMP-2-induced transcriptional activity of Smad5, whereas Smad3 alone had no effect. Moreover, overexpression of the dominant-negative mutant, Smad3ΔC (43), and menin, augmented BMP-2-induced transcriptional activity of the Smad5 response element containing the 3GC2-lux reporter construct (Fig. 9A). These findings suggest that the interaction of menin with Smad3 negatively regulates BMP-2 signaling. Finally, we examined the effects of the menin-Smad3 interaction on the transcriptional activity of Runx2 in MC3T3-E1 cells. Although menin or Smad3 alone did not affect the expression of the Runx2 response element containing OSC-luci reporter construct, the combination of menin and Smad3 antagonized BMP-2-induced luciferase activity in Smad5- and/or Runx2-transfected cells (Fig. 9B). Taken together, the data suggest that the interaction of menin and Smad3 negatively regulates the BMP-2-Runx2 cascade in cells committed to the osteoblast lineage.

**DISCUSSION**

In the present study, we have examined mechanisms involved in the differential effects of menin in regulating osteogenesis and osteoblast differentiation. The novel insights provided are 2-fold. First, the key role of menin and its interactions with the BMP-Runx2 cascade in commitment of mesenchymal stem cells to the osteoblast lineage is revealed. Second, the cross-talk between BMP-2 and TGF-β-signaling pathways critical for modulating the extent of osteoblast differentiation is shown to be integrated via menin.

Runx2, a target of BMP-2 and TGF-β, is the pivotal osteoprogenitor transcription factor directing the expression of bone matrix proteins such as collagen type I, osteopontin, OCN, and others (43, 44). In mesenchymal stem cells, represented here by the bone marrow ST2 cells, inactivation of menin antagonized BMP-2 induction of Runx2 expression and osteoblast differentiation markers such as ALP activity and OCN expression. Menin interacted both physically and functionally with the BMP-2 signaling molecules, Smad1/5, and menin inactivation led to a reduction in the BMP-2 directed transcription of a Smad1/5-responsive gene promoter in the ST2 cells. Cooperation between Runx2 and the BMP-2, R-Smad, and Smad5, has been noted previously to induce osteoblast-specific gene expression in mesenchymal C2C12 cells (38). In the present study, we observed that menin interacted both physically and functionally with Runx2 in ST2 cells and that inactivation of menin antagonized the activity of the transcription factor. Our previous studies showed that menin promotes the commitment of multipotential mesenchymal stem cells to the osteoblast lineage, and the present studies suggest that this occurs through the BMP-2-Smad1/5-Runx2 cascade.

In contrast, important differences were noted in the mecha-
nism of action of menin in the differentiated osteoblasts. In part this was to be expected given that previously we had observed in either C3H10T½ mesenchymal stem cells that had undergone differentiation into osteoblastic cells by treatment for 14 days with BMP-2, or the MC3T3-E1 osteoblast cells, that menin anti-sense oligonucleotides no longer affected the response of differentiation markers to BMP-2 (22). This suggested that the role of menin as a positive differentiation factor decreases as osteoblastic differentiation progresses. Furthermore, in MC3T3-E1 cells stably expressing antisense menin RNA, the expression of differentiation markers and mineralization were increased compared with vector-alone transfected control cells indicating that menin serves as an inhibitor of osteoblastic maturation after the commitment into osteoblasts (22).

In the present study, although menin interacted physically with Smad1/5 in osteoblasts, it was no longer essential for the BMP-2 signaling molecules with respect to their full transcriptional activation function. The precise mechanism underlying the lack of a transcriptional effect is not known. However, there is precedent for R-Smad interaction with protein partners resulting in altered transcriptional activity under some circumstances, but not others. For example, Liberati et al. (45) found that mutations in the MH1 domain of Smad3 did not disrupt binding of Smad3 with the histone deacetylase activity (HDAC1) associated proteins, c-ski, SnoN, and transforming growth-interacting factor, but did abrogate the ability of Smad3 to repress gene transcription. These findings suggested that additional protein mediators are needed for the Smad3-associated histone deacetylase activity and in turn the suppressive effect of Smad3 on gene transcription. Likewise, other protein mediators may be needed for the menin-Smad1/5-induced suppression of gene transcription. If so, it could be that these protein mediators are expressed in the multipotential mesenchymal ST-2 cell but not the committed osteoblast MC3T3-E1 cell.

Menin interacted neither physically nor functionally with Runx2 in the MC3T3-E1 cells. In contrast, menin interacted with TGF-β, R-Smad, and Smad3 in these osteoblast cells, and, as a consequence, suppressed BMP-2-induced transcriptional activities of Smad1/5 and Runx2. This would be consistent with the report of Alliston et al. (46) that Smad3 suppressed the expression of Runx2 and its transcriptional activity in...
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Fig. 10. Schematic model for regulation of the BMP-Runx2 cascade by menin in osteoblast differentiation. Menin promotes the commitment of multipotential mesenchymal stem cells to the osteoblast lineage through interactions with Smad1/5 and Runx2, whereas the interaction of menin and Smad3 inhibits later osteoblast differentiation by negatively regulating the BMP-Runx2 cascade after the commitment to the osteoblast lineage.

MC3T3-E1 cells. In addition, we have shown previously that Smad3 suppressed the expression of OCN in this cell line (47), likely as a consequence of the reduced expression of Runx2 as shown here.

Our present understanding of the differential role of the BMP-Smad-Runx2 cascade in moving from uncommitted mesenchymal stem cells to committed osteoprogenitor to differentiated osteoblast is schematized in Fig. 10. In the mesenchymal stem cell, BMP-2 activates R-Smads, Smad1/5, and menin interacts with Smad1/5 physically and functionally, resulting in increased expression and activation of Runx2. Moreover menin interacts with Runx2 physically and functionally, promoting the commitment of the multipotential mesenchymal stem cells to the osteoblast lineage.

We demonstrated that menin inactivation did not influence the nuclear accumulation of Smad1/5 and Runx2 in ST2 cells. In addition, we showed that the interaction of menin with its protein partners occurred in the nucleus not the cytoplasm. Hence, the effects of menin are likely to be exerted in the nucleus, probably by influencing the interactions of menin with cofactors that alter the efficacy of transcriptional regulators of genes important for osteoblast differentiation. The co-activator P300/CBP, the cyclic AMP response element-binding protein, and the three runt domain-containing transcription factors (Runx2), that all affect osteoblast function, interact with R-Smads of both the BMP and TGF-β signaling pathways. Smad3 interacts with transcription factors c-Fos, NF-κB, glucocorticoid receptor, and vitamin D receptor, which again play all critical roles in osteoblast function. Moreover, in the Wnt signaling pathway, important for bone formation, β-catenin and Lef1/Tcf interact with Smads. Among all these molecules, P300/CBP is the one that interacts with both the Smads and Runx2. Smad3 binding to P300/CBP cooperatively regulates target gene transcription (48–52), and in ROS 17/2.8 osteoblast cells, regulation of the OCN gene by P300/CBP requires Runx2 (53). However, prior to the present study, little has been reported with respect to the BMP or TGF-β signaling pathways and interactions of Smads and Runx2 and osteoblast differentiation. Thus, the studies described here lay the groundwork for future studies to identify other protein players such as those listed above in these signaling pathways.

After commitment to the osteoblast lineage, the role of menin changes and it no longer exerts a positive influence on the BMP-2/Smad1/5/Runx2 pathway. Indeed, in our previous study, inactivation of menin promoted the expression of Runx2 leading to increased collagen type I and OCN expression, ALP activity, and mineralization in MC3T3-E1 osteoblast cells (22). In addition, as shown in the present study, and schematized in Fig. 10, menin is important for the effect of TGF-β-activated Smad3 in inhibiting the BMP-2/Runx2 cascade. Divergent effects of BMP and TGF-β have been observed previously in osteoblasts (31, 38, 54). This raises the issue of cross-talk between BMP and TGF-β-signaling pathways that could occur in different ways. Some co-activators of Smads (e.g. FAST, Mix proteins, e-Jun, etc.) seem to mediate their regulatory activities by binding specific DNA sites and targeting a particular subset of TGF-β/BMP-responsive genes (55). Recently Lee et al. (56) reported that BMP-2-induced Runx2 expression is mediated by the bone-inducing transcription factor, Dlx5, which is expressed at a late stage of differentiation, and that TGF-β opposes BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. In our study, the data suggest that BMP- and TGF-β-signaling pathways cross-talk via menin.

A number of issues remain to be clarified with respect to the stage prior to the commitment of the mesenchymal stem cell to the osteoblast lineage. First, what is the role of TGF-β signaling? Is it able by itself to stimulate osteogenesis? Second, does menin interact with Smad3 in multipotential stem cells, and if so, what are the consequences of the interaction on the BMP-2/Runx2 cascade? These issues are presently under investigation in our laboratories.

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Hideaki Sowa, Hiroshi Kaji, Geoffrey N. Hendy, Lucie Canaff, Toshihisa Komori, Toshitsugu Sugimoto and Kazuo Chihara

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