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Menin and TGF-β Superfamily Member Signaling via the Smad Pathway in Pituitary, Parathyroid and Osteoblast

Abstract

Pituitary: Menin is a *Smad3*-interacting protein; inactivation of menin blocks transforming growth factor (TGF)-β and activin signaling, antagonizing their growth-inhibitory properties in anterior pituitary cells. Menin is also required for the activin-induced inhibition of prolactin expression mediated by the Smads and the transcription factor, Pit-1. The interaction between menin and *Smad3* is direct. **Parathyroid:** In cultured parathyroid cells from uremic hemodialysis patients, in which the menin signaling pathways are probably still intact, menin inactivation achieved by menin antisense oligonucleotides leads to loss of TGF-β inhibition of parathyroid cell proliferation and parathyroid hormone (PTH) secretion. Moreover, TGF-β does not affect the proliferation and PTH production of parathyroid cells from multiple endocrine neoplasia type 1 (MEN1) patients. **Osteoblast:** *Men1*-null mouse fetuses that die at day 12 or earlier have cranial/facial hypoplasias implicating menin in bone development. Menin is required for the commitment of multipotential mesenchymal stem cells into the osteoblast lineage. This is achieved by menin interacting physically and functionally with bone morphogenetic protein (BMP)-2 regulated Smads, such as *Smad1* and *Smad5*, and the key osteoblast regulator, *Runx2*. These interactions are lost as the committed osteoblasts differentiate further at which time menin interacts with *Smad3*, mediating the negative regulation of *Runx2* by TGF-β. Menin also suppresses osteoblast maturation, partly by inhibiting the differentiation actions of *JunD*.

Key words
Menin · Parathyroid · Anterior pituitary · Osteoblast

Menin

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by endocrine tumors of the parathyroid, anterior pituitary and pancreatic islets. The *MEN1* gene [1] encodes a protein of 610 amino acids – menin – located predominantly in the nucleus [2,3]. In *MEN1* carriers, inactivating mutations give rise to a truncated product consistent with menin acting as a tumor suppressor [4]. The biological properties and roles that menin plays physiologically and the mechanisms by which menin loss leads to tumorigenesis in select tissues are unclear, but are currently under intensive investigation.

Mice heterozygous for genetic ablation of the *Men1* gene develop endocrine tumors during their lifetime similar to human MEN1 patients [5,6]. Interestingly, homozygous deletion of *Men1* is lethal in the embryonic stage, with fetuses dying at mid-gestation with defects in multiple organs [7]. Menin has been shown to be an essential component of particular histone methyltransferase complexes that control *Hox* gene expression, which is important

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for early development [8,9]. It may be involved in telomere biology [10,11] and DNA replication and repair [12,13].

Menin interacts with several transcription regulators including Smads, *JunD* and NF-κB, and modulates their activities [14]. The focus of this review will be on menin's interaction with Smads and the relevance for TGF-β superfamily member signaling with respect to control of pituitary and parathyroid cell proliferation (and its dysregulation providing a stimulus to neuroendocrine tumorigenesis if menin activity is lost [15]). In addition, this review will deal with the role that menin plays in osteoblastogenesis and osteoblast differentiation.

The TGF-β Signaling Pathway

TGF-β signals through type I and type II serine/threonine kinase cell membrane receptors (Fig. **1**). The type II receptor is constitutively phosphorylated and, when activated by binding of the TGF-β ligand, recruits and transphosphorylates the type I receptor. The activated type I receptor phosphorylates the receptor-regulated Smads (*Smad2* and *Smad3*) and their association with the common partner, *Smad4*. The *Smad3*/*Smad4* complex then translocates to the nucleus to activate specific genes [16,17].

The cytokine TGF-β causes growth inhibition of most cell types; loss of TGF-β signaling may push the cell towards inappropriate growth that ultimately results in tumor formation. One way that some oncoproteins abrogate normal cellular growth control is by blocking TGF-β signaling. Inactivating mutations in various components of the TGF-β /Smad signaling pathway are found in numerous types of human cancer. The TGF-β type II receptor is often inactivated by mutation in colon and gastric cancers with microsatellite instability [18]. Deletion or point mutation in the TGF-β type I receptor gene is found in pancreatic, biliary, colorectoral, cervical, and breast cancers [19 – 22]. The tumor suppressor gene, *Smad4*, is mutated in nearly half of all pancreatic cancers [23] and to a lesser extent in other types, such as esophageal adenocarcinoma [24], head and neck carcinoma [25], hepatocellular carcinoma [26], neuroblastoma [27], breast cancer [28] and lung cancer [29]. While no mutations in the *Smad3* gene have been reported to date, the *Smad2* gene is mutated in colon, head and neck, as well as lung carcinomas [30 – 32]. We hypothesized that menin, acting as a tumor suppressor, may be involved in the growth inhibitory actions of TGF-β in endocrine cells.

Menin and Anterior Pituitary Cells

In the rat anterior somatolactotrope GH4C1 cell line, TGF-β stimulates menin expression rapidly and dose-dependently [33]. To explore the involvement of menin in the TGF-β-signaling pathway, GH4C1 cells were stably transfected with antisense menin cDNA (AS); markedly reduced menin expression was demonstrated. TGF-β's ability to stimulate menin accumulation was blocked in the AS cells. In addition, antisense menin antagonized the normal inhibitory effect of TGF-β on cell proliferation as measured by cell number, tritiated thymidine uptake and cell viability assays [33].

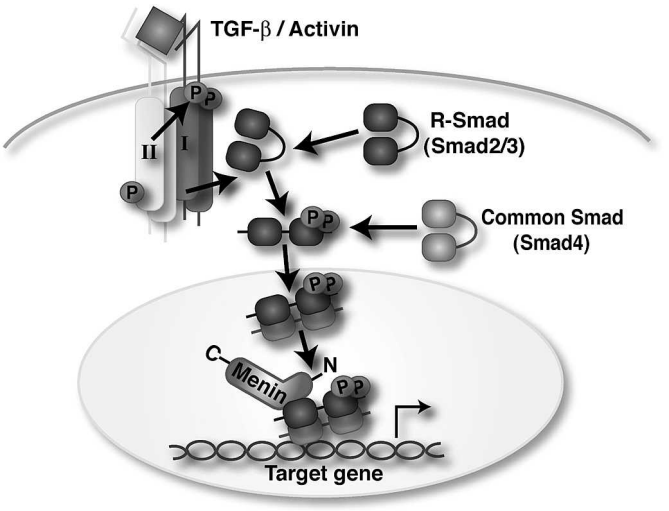

Fig. **1** Menin and TGF-β /Activin signaling pathway. TGF-β and activin signal through their Type I and Type II serine/threonine kinase receptors. Receptor-mediated phosphorylation of *Smad2* or *Smad3* induces their association with the common partner *Smad4* followed by translocation into the nucleus, where these complexes activate transcription of specific genes. In the nucleus, menin is bound to *Smad3* (but not *Smad2* or *Smad4*) and facilitates transcription by the Smad complex.

Menin Inactivation Inhibits TGF-β-induced Transcriptional Activity

We examined the effects of menin antisense on TGF-β-mediated transcriptional responses. For this, we used the human hepatoma HepG2 cell line that has been well-characterized with respect to TGF-β effects. TGF-β greatly increased luciferase activity in the absence of antisense menin in these cells when transfected with the TGF-β-responsive plasminogen activator inhibitor-1 (PAI-1) promoter luciferase reporter gene construct (3TP-Lux). However, reduced menin expression achieved by antisense menin co-transfection markedly blocked TGF-β-induced transcriptional activity. The specificity of the response was demonstrated by the restoration of transcriptional activity with co-transfection of increasing amounts of a sense menin construct [33].

Menin Interacts with Smad3

To examine whether menin would interact with any of the Smad proteins, COS (African green monkey kidney) cells were transiently transfected with cDNA coding for epitope-tagged *Smad2*, *Smad3* or *Smad4* in the presence or absence of menin cDNA. Menin specifically co-immunoprecipitated with *Smad3*, but not with *Smad2* or *Smad4* [33]. Both *Smad2* and *Smad3* co-immunoprecipitated with *Smad4* in response to TGF-β, confirming the functionality of our system.

To examine whether the interaction is direct, we performed GST-*Smad3* pull-down reactions with *in vitro* transcribed/translated full-length and deletion mutants of menin [34]. These studies showed that the interaction between menin and *Smad3* is direct, and that the sequence between amino acids 101 – 195 is critical for this. GST-menin pull-down reactions with full-length and deletion mutants of *Smad3* demonstrated that the *Smad3* MH2 do-

main and phosphorylation of the COOH-terminus are not essential for *Smad3*-menin interaction [34]. Further mapping studies are underway to localize the exact interacting regions in both proteins.

Reduced Menin Expression Impairs Smad3-mediated Transcriptional Activity

To explore the functional relationship of *Smad3* and menin, we examined whether antisense menin would affect *Smad3*-mediated transcriptional activity. *Smad3* overexpression in either HepG2 or GH4C1 cells increased transcriptional activity of the transfected 3TP-Lux construct (described above) itself and augmented TGF-β-induced transcriptional activity, and antisense menin significantly inhibited the *Smad3*-mediated transcriptional activity. The transcriptional activity was restored by co-transfection of the sense menin construct. These data suggest that menin functionally interacts with *Smad3*, and that menin inactivation blocks *Smad3*-mediated transcriptional effects in the TGF-β pathway [33]. In addition we have demonstrated that reduced menin expression achieved by the antisense technique does not affect TGF-β-induced *Smad3* and *Smad4* oligomerization and nuclear translocation [33].

Reduced Menin Expression Disrupts Smad3 Binding to DNA

The *Smad3/Smad4* complex recognizes specific binding sites on gene promoters. We examined whether antisense menin would affect the DNA binding ability of the *Smad3/Smad4* complex by using an electromobility shift assay (EMSA) with nuclear extracts from GH4C1 cells and a probe derived from the same promoter, that for PAI-1, used for the TGF-β responsive promoter reporter gene experiments. Levels of *Smad3/Smad4*-DNA complex were decreased when menin levels were reduced by antisense menin transfection with *Smad3* and *Smad4*. Co-expression of sense menin restored the levels of the Smad-DNA complex. Therefore, menin interacts with the TGF-β pathway in the nucleus through *Smad3* and inactivation of menin disrupts *Smad3* binding to its cognate DNA element, thereby blocking TGF-β signaling [33].

Thus, menin plays an important role in supporting TGF-β and *Smad3* transcriptional control of cell growth, and reduced menin expression disrupts TGF-β-mediated transcription and growth inhibition. Some oncoproteins, SkiI and SnoN, inhibit the TGF-β signaling pathway through a Smad transcriptional co-repressor [35, 36]. Menin may act by blocking the effect of co-repressors, facilitating TGF-β signaling.

Pituitary adenomas are common, but in contrast to sporadic parathyroid tumors and enteropancreatic tumors, mutation of the *MEN1* gene is not a major contributor to sporadic pituitary tumorigenesis. However, variable and decreased expression of the menin protein was noted by immunohistochemistry in a series of sporadic pituitary adenomas [37]. Therefore, it is likely reduced menin expression is contributing to the more common sporadic pituitary tumorigenesis.

Activin, another member of the TGF-B superfamily, plays an important role in regulating anterior pituitary gland function acting on gonadotropes, somatotropes and lactotropes. Activin is a negative regulator of cell growth and prolactin (PRL) production in pituitary lactotrope cells [38]. We have demonstrated that inactivation of menin achieved through three different antisense technologies (cDNA antisense, oligonucleotide antisense, and siRNA) blocks activin signaling via the Smads in somatolactotrope cells [39]. This results in an increase in *PRL* gene expression, *Pit1* gene expression, and the loss of pituitary cell growth inhibition by activin.

Menin and Parathyroid

Several local growth factors such as endothelin-1, TGF-α, fibroblast growth factors, and insulin-like growth factors are expressed in parathyroid cells and in some cases have been shown to stimulate the proliferation of parathyroid cells [40]. The expression of TGF-β and its role in parathyroid cell function has not previously been studied. We have demonstrated that TGF-β is expressed in the parathyroid, predominantly by the endocrine cells, and that TGF-β negatively regulates the proliferation and PTH production of these cells [40].

A role for alterations in the *MEN1* gene has been clearly established in the pathogenesis of sporadic primary parathyroid tumorigenesis. It has also been shown that a substantial number of "hyperplastic" tumors from patients with uremic refractory hyperparathyroidism are monoclonal in nature. However, allelic loss of chromosome 11 markers and/or *MEN1* gene inactivation has been demonstrated in only a very few of uremic types of tumor (see ref. [40] and its references). Thus, *MEN1* gene abnormality rarely plays a role in the clonal emergence in uremic parathyroid hyperplasia. Given this background, our study used tissue from uremic hyperparathyroidism patients as an appropriate surrogate for normal human parathyroid tissue in which the *MEN1* gene is not impaired [40].

Addition of TGF-β to parathyroid cells from patients with secondary hyperparathyroidism inhibited their proliferation and PTH secretion. These responses to TGF-β were lost when menin was specifically inactivated by antisense oligonucleotides [40]. Moreover, TGF-β did not affect the proliferation and PTH production of parathyroid cells from MEN1 patients. Therefore, TGF-β is an important autocrine/paracrine negative regulator of parathyroid cell proliferation, and PTH secretion and loss of TGF-β signaling due to menin inactivation may contribute to parathyroid tumorigenesis.

Chromosome 15q, where the *Smad3* gene is located, has been implicated as the site of an as yet unidentified tumor suppressor in endocrine tumors. A significant proportion of both parathyroid and enteropancreatic tumors show loss of heterozygosity of DNA markers surrounding the *Smad3* locus [41]. However, no acquired clonal mutations have been identified in the *Smad3* protein-coding exons in such tumors [41]. Other mechanisms that could lead to altered *Smad3* expression in the tumors have yet to be examined. Also, intriguingly, *Smad3* can serve as a transcriptional co-activator [42, 43] of the vitamin D receptor (VDR)

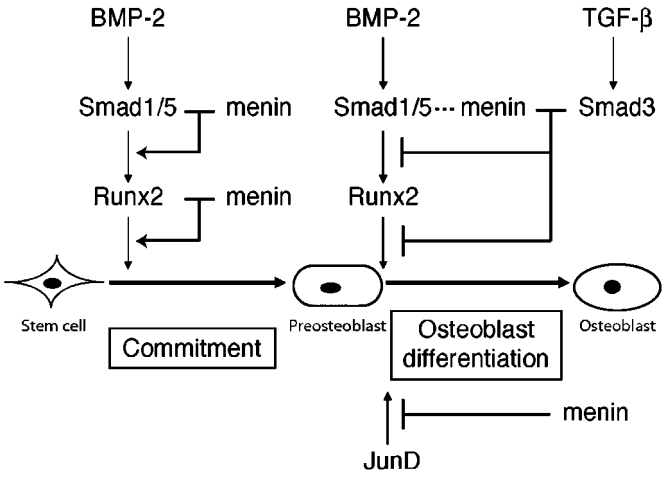

Fig **2** Schematic model for regulation of osteoblast differentiation by menin. Menin promotes the initial commitment of multipotential mesenchymal stem cells to the osteoblast lineage through interactions with *Smad1*/*Smad5* and *Runx2*, whereas the interaction of menin and *Smad3* inhibits later osteoblast differentiation by negatively regulating the BMP-*Runx2* cascade – for example, via TGF-β/*Smad3* signaling. In addition, menin antagonizes the later osteoblast differentiating actions of *JunD*.

that mediates inhibition of parathyroid cell proliferation by 1,25-dihydroxyvitamin D [44]. Therefore, one way or the other, either direct gene mutation or epigenetic alteration, menin, *Smad3* and the VDR probably contribute to dysregulated parathyroid cell proliferation and tumorigenesis.

Menin and Bone

Both TGF-β and BMPs, which are primarily synthesized by osteoblasts, play an important role in bone formation and remodeling [45]. *In vivo*, local administration of TGF-β alters expression of many genes regulating osteoblast activity, induces synthesis of matrix proteins and increases bone formation. BMP2 (amongst several others) is essential for osteoblastic growth and differentiation as well as bone formation.

Homozygous menin inactivation in mice is embryonic lethal and some fetuses exhibit clear defects in cranial and facial development [5]. Heterozygous menin knockout mice are of normal phenotype (as are humans harboring a germline inactivating mutation in one *MEN1* gene allele) indicating haplosufficiency with respect to this particular function of menin [5,7]. Since cranial bones are formed by intramembranous ossification, these findings suggested that menin might play an important role in bone formation (Fig. **2**). We have demonstrated that menin is required for the commitment of multipotential mesenchymal stem cells to the osteoblast lineage [46]. This occurred, in part, by the roles played by menin in facilitating BMP signaling via Smads and the transcriptional activity of the key osteoblast regulator, *Runx2* [46,47]. Menin interacted physically and functionally with *Smad1*, *Smad5* and *Runx2* in mesenchymal stem cells [47]. In committed osteoblasts, these interactions were, for the most part, lost and menin inhibited later osteoblastic differentiation. This occurred, in part, through menin interacting with the TGF β /*Smad3* pathway [47]. In committed osteoblasts, AP-1 factor *JunD* promotes differentiation and interacts with menin, both physically and functionally [48]. Menin also suppresses osteoblast maturation, partly by inhibiting the differentiation actions of *JunD* [48].

Perspective

One report has provided preliminary evidence that embryonic fibroblasts from *Men1*$^{-/-}$ mice are less responsive to TGF-β [49]. Further work with these cells as well as tissue-specific *Men1* knockout mice are likely to provide additional insights into the roles played by menin in TGF-β superfamily member signaling.

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