Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized primarily by endocrine tumors of the parathyroids, anterior pituitary, and enteropancreatic endocrine tissues. Affected individuals carry a germ-line loss-of-function mutation of the MEN1 gene, and tumors arise after loss of the second allele. Homozygous loss of Men1 in the germ line of mice results in early embryonic lethality, with defective development of neural tube, heart, liver, and craniofacial structures. We generated immortalized wild-type (WT) and menin-null mouse embryo fibroblast (MEF) cell lines and evaluated their characteristics, including global expression patterns. The WT and menin-null cell lines were aneuploid, and the nulls did not display tumorigenic characteristics in soft agar assay. Expression arrays in menin-null MEFs revealed altered expression of several extracellular matrix proteins that are critical in organogenesis. Specifically, transcripts for fibulin 2 (Fbln2), periostin (Postn), and versican (chondroitin sulfate proteoglycan (Cspg2)), genes critical for the developing heart and known to be induced by transforming growth factor-3 (TGF-3), were decreased in their expression in menin-null MEFs. Fbln2 expression was the most affected, and the reduction in menin-null MEFs for Fbln2, Postn, and Cspg2 was 16.18-, 5.37-, and 2.15-fold, respectively. Menin-null MEFs also showed poor response to TGF-3-induced Smad3-mediated transcription in a reporter assay, supporting a role for menin in this pathway. Postn and Cspg2 expression in WT, unlike in null MEFs, increased on TGF-3 treatment. The expression changes associated with the loss of the tumor suppressor menin provide insights into the defective organogenesis observed during early embryonic development in Men1-null mouse embryos. (Mol Cancer Res 2007;5(10):1041–51)

Introduction

Multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant disorder, is characterized by multiple tumors of the parathyroid, anterior pituitary, and enteropancreatic endocrine tissues. At a lower penetrance, foregut carcinoids and adrenal tumors also can occur, as well as hormone nonproducing tumors, such as facial angiofibroma, truncal collagenoma, lipoma, leiomyoma, meningioma, and ependymoma (1). Loss-of-function mutations in the tumor suppressor gene MEN1 are responsible for the syndrome (2). Biallelic somatic mutations in the MEN1 gene are also often found in sporadic tumors of endocrine tissues, such as parathyroid adenoma, gastrinoma, insulinoma, and foregut carcinoid (3). The MEN1 gene encodes a 610-amino acid protein, which is ubiquitously expressed and resides primarily in the nucleus (4). The amino acid sequence of the protein, denoted menin, does not provide any clues as to its function.

Several proteins that bind menin directly or indirectly have been reported. Many of these are nuclear proteins involved in transcriptional regulation, including JunD, nuclear factor-κB, Pem, the COMPASS-like complex, Smad proteins 1, 3, and 5, RunX2, and histone deacetylases (5). The COMPASS-like complexes include the trithorax proteins mixed lineage leukemia 1/2, which possess histone methyl transferase activity. The menin-histone methyl transferase complexes methylate lysine 4 of histone H3 (H3K4), which is generally associated with activation of transcription (6, 7). Although the precise biochemical function of menin in this complex needs to be elucidated, the importance of menin in this complex is apparent from the observation that the loss of menin results in reduced H3K4 methylation and associated reduction in the expression of
known mixed lineage leukemia targets Hoxc6 and Hoxc8 (6). Interaction of menin with Smad3 is reported to result in activation of transforming growth factor-β (TGF-β)-induced transcription (8), and the interaction of menin with Smad1, Smad3, Smad5, and Runx2 may participate in the TGF-β-mediated and bone morphogenetic protein-2–mediated pathways of commitment of multipotential mesenchymal stem cells to osteoblast lineage (9-11). Previous studies of gene expression in tumors, cell lines, and embryos have identified nonoverlapping sets of gene(s), whose expression is affected by menin (5, 12, 13). A recent report of chromatin immunoprecipitation–on-chip analysis has identified thousands of menin-binding promoter targets in the human genome (14).

The clinical features of the MEN1 syndrome are well emulated in the mouse Men1 model. Mice heterozygous for Men1 alleles, on loss of the remaining allele as they age, develop endocrine tumors resembling the human MEN1 condition (15, 16). Homozygous loss of Men1 alleles results in embryonic lethality (E11.5-E13.5), indicating a requirement for menin in early development. Before demise, menin-null embryos are smaller in size than WT, often with body hemorrhages and edema, and a substantial portion of embryos reveal defects in neural tube closure as well as abnormal cardiac and liver development (15, 17).

Lack of cell lines from MEN1 tumors has been an impediment in evaluation of the molecular consequences of the loss of menin. To gain insight into the basic biological function of menin, we generated and characterized multiple mouse embryo fibroblast (MEF) cell lines lacking menin. Expression changes associated with the loss of menin provide insight into the importance of menin in early embryonic development.

Results and Discussion

Characterization of Menin Wild-Type and Menin-Null MEF Cell Lines

The development of a mouse Men1 knockout model provided an opportunity to establish menin-null cell lines and to gain insight into the biological function(s) of menin. Mouse embryos null for both Men1 alleles do not survive beyond E11.5 to E13.5 (15). Therefore, MEF cell lines of wild-type (WT) and nullizygous genotype were established from early-stage (E9.5) embryos obtained by crossing mice heterozygous for the Men1 alleles (Men1 ΔN3-8/+). A total of four WT and six nullizygous (menin-null) cell lines as well as five subclones (N41.1-N41.5) of the menin-null cell line N41 were established. Four WT (W10, W26, W46, and W50) and four menin-null (N12, N17, N40, and N47) cell lines were used for characterization and expression analysis in this study. Both male (W10, W46, N12, and N40) and female (W26, W50, N17, and N47) embryos evenly represented the WT and menin-null MEFs. The presence or absence of Men1 expression, respectively, in WT and menin-null cell lines was shown at both the RNA and protein levels (Fig. 1A and B). Both reverse transcription-PCR (RT-PCR) for the Men1 coding region as well as a Western blot with menin antibodies against a COOH-terminal peptide showed the absence of Men1 expression in menin-null MEFs (Fig. 1A and B).

Several MEF cell lines from WT (W10, W46, and W50) and menin null (N12, N17, N40, N47, and N41.4) were analyzed by spectral karyotyping (SKY) to identify any discernible chromosomal changes associated with the loss of menin. All the cell lines were found to be aneuploid, primarily tetraploid, although the cell lines were heterogeneous in chromosome number. Translocations were also seen in both genotypes; however, there was no consistent variation that could be associated with the homozygous loss of menin (data not shown).

We tested whether MEFs lacking menin would be tumorigenic by evaluating their ability to form colonies in soft agar. Five menin-null cell lines (N12, N17, N40, N47, and N41.4) along with the four WT (W10, W26, W46, and W50) were tested. None were capable of forming colonies in soft agar with the exception of N41.4 (a subclone of N41), which formed a few smaller colonies (data not shown). Thus, these menin-null cell lines in general did not show tumorigenic characteristics in culture.

Effect of TGF-β on Menin-Null Cell Lines

Previous studies have shown that menin interacts with Smad3 and enhances TGF-β-induced transcriptional activation (8). We evaluated whether the menin-null cell lines lacking menin would respond poorly to TGF-β by using a Smad3-mediated, TGF-β-responsive luciferase reporter assay. The plasminogen activator inhibitor-1 gene promoter contains Smad3-binding sites. A luciferase reporter gene construct driven by this promoter (3TP-Lux) is expected to be activated by TGF-β. This construct was transfected into five menin-null and four WT MEF cell lines and the luciferase activity was quantitated with and without TGF-β treatment. The ratio of luciferase activity in the presence and absence of TGF-β (Fig. 1C) indicates that WT cells generally respond better than the menin-null cell lines (P < 0.05). Transient expression of menin in menin-null MEFs increases the luciferase activity (Fig. 1D), suggesting that the deficiency may be due to the absence of menin. This supports the prior observation that reduction in menin results in reduced response to TGF-β. Thus, the menin-null MEFs are suitable to explore the TGF-β-induced menin-mediated expression changes.

Expression Changes Associated with Menin Loss

As a nuclear protein that interacts with many other factors that modify transcription, menin presumably affects gene expression of many targets. Establishment of the spontaneously immortalized MEF cell lines lacking menin allowed for a thorough investigation of transcriptional changes. The spontaneously immortalized cell lines were aneuploid, so we chose multiple cell lines (four or more for WT and menin null each) that would reduce the effect of clonal variations. RNA isolated from WT and four menin-null MEF cell lines was labeled with Cy5 and Cy3 fluorochrome and hybridized to spotted arrays consisting of 21,120 cDNA clones representing 16,000 unique mouse transcripts. A total of 32 pairwise hybridizations were carried out between four WT and four menin-null cell lines, all with a dye swap protocol. In addition, two hybridizations with pooled WT cell lines against pooled menin-null cell lines, and three self-self hybridizations, were done for comparison purposes. On average, we obtained 15,082 (of
21,120) positive probes when we required both Cy3 and Cy5 channel intensity (background subtracted signals) to be >200. All the data from the expression arrays are deposited at the National Center for Biotechnology Information\(^5\) and the Gene Expression Omnibus accession number is GSE6513. By requiring 2-fold or greater changes in the expression ratio (median ratio over all 32 hybridizations), and a Benjamini-Hochberg adjusted \(P\) value (\(t\) test) of <0.01, we obtained in menin-null MEFs 66 and 70 underexpressed and overexpressed genes, respectively. By eliminating redundant and probes with no annotation, we further reduced the unique probes to 54 and 56 for underexpression and overexpression (Figs. 2 and 3). As illustrated in these two figures, the majority of these genes also showed high expression ratio in pooled WT versus menin-null hybridizations (35 of 54 genes in Fig. 2 have expression ratios >2-fold in both pooled experiments, and first 20 genes are the same except Matn2). The expression level in three self-self control experiments showed no significant changes (expression ratio, 1.10 ± 0.16). We then analyzed the Gene Ontology function enrichment by using the EASE program\(^6\) by submitting all genes in Figs. 2 and 3 and all genes printed in the array. Enriched ontology terms (extracellular space/matrix, cell adhesion, receptor and receptor activity, protein binding, and development) were annotated in Figs. 2 and 3 with the \(P\) values obtained from EASE. The entire \(P\) value table is provided in Supplementary Table S1. Thus, lack of menin resulted in altered expression of several genes encoding extracellular space/matrix proteins.

\(^5\) http://www.ncbi.nlm.nih.gov/geo/

\(^6\) http://david.niaid.nih.gov/david/ease.htm

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**FIGURE 1.** Absence of Men1 gene expression (A and B) and reduced response to TGF-\(\beta\) in menin-null MEFs (C and D). A and B. Expression of Men1 in total RNA was analyzed by RT-PCR (A) and in whole-cell lysates by Western blotting using menin antibodies (B) in four WT (W10, W26, W46, and W50) and four menin-null (N12, N17, N40, and N47) cell lines. RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and Western blotting with \(p84\) antibodies were used as controls. C. Luciferase reporter assay for a TGF-\(\beta\)-inducible plasminogen activator inhibitor-1 promoter in four WT (W10, W26, W46, and W50) and five menin-null (N12, N17, N40, N41.4, and N47) MEFs. Cells were transfected with the 3TP-Lux reporter construct and treated with (or without) TGF-\(\beta\), and the luciferase activity was measured in cell lysates. The ratio of enzyme activity observed in the presence and absence of TGF-\(\beta\) is reported. Each point represents an average data from six wells. A two-tailed \(t\) test indicates that there is a significant difference in the response of WT to menin-null cell lines (\(P < 0.05\)). Gray columns, menin-null cell lines; black columns, WT cell lines. D. Luciferase reporter assay for a TGF-\(\beta\)-inducible plasminogen activator inhibitor-1 promoter in menin-null (N41.4 and N47) MEFs on transient expression of menin. The experiment is carried out as in C except that the cells were cotransfected with Men1 expression construct (black columns) or the vector (gray columns); the increasing amount (0.5 and 1 \(\mu\)g) of expression construct and the vector transfected is indicated. The luciferase activity relative to the internal transfection control (Renilla luciferase) is reported. The Western blot analysis (bottom) shows that the whole-cell lysates of N47 cell line transfected with the Men1 expression construct express the menin protein.
Validation of Expression Changes

We confirmed the changes in expression of several transcripts independently by Northern blot, Western blot, and quantitative PCR. The two genes that showed the maximum change in menin-null cell lines were the underexpression of fibulin 2 (Fbln2) and overexpression of heat shock protein b1 (Hspb1/Hsp25; Figs. 2 and 3). Northern blot results were consistent with the array data for Fbln2 and Hspb1 (Fig. 4A and B), although there were some clonal variations in expression. Reduced expression in menin-null cells of another extracellular matrix protein gene periostin (Postn) was also verified by Northern and Western blot analysis (Fig. 4A and C). We also looked at the increased expression in menin-null MEFs of Cd24a protein, a cell surface marker, by fluorescence-activated cell sorting analysis of cells stained with antibodies for Cd24a (Fig. 4D). The results indicate that the menin-null cell lines generally express higher levels of Cd24a compared with the WT cell lines, although the W10 cell line turned out to be an exception, as was apparent from Fig. 3; quantitative PCR results also show unusual high Cd24a expression in W10 (see Fig. 5B).

We also evaluated expression of 14 different transcripts by quantitative PCR. RNA from individual cell lines as well as pooled WT and menin-null RNA samples (using equivalent amounts of RNA from each of the four cell lines) were evaluated. Expression in pooled WT and menin-null cells, as well as representation of the same data in terms of relative changes between WT and menin null, is shown in Fig. 5A: Cspg2, Igfbp3, Wnt5a, Pbx3, Sh3kbp3, Sgk, and Ccnb2 represented those that were lower in menin null and Btg2, Peg3, Cp, Vif2, and Chl represented the ones higher in menin nulls. The fold difference observed for most of these transcripts from the array data was consistent with that observed by quantitative PCR. Quantitative PCR for Mox2,
Cd24a, Cspg2, and Btg2 in eight individual MEF cell lines is also shown (Fig. 5B) and was generally consistent with the array data.

**TGF-β–Induced Menin-Mediated Gene Expression Changes**

We treated a WT (W10) and a menin-null (N47) MEF cell line with varying amounts (0, 2.5, 5, and 10 ng/mL) of TGF-β for varying lengths of time (12, 24, and 48 h), and expression of the three extracellular matrix proteins, Postn, Cspg2, and Fbln2, was evaluated (Fig. 6). RT-PCR, quantitative RT-PCR, and Western blot analysis indicate that Postn was induced by TGF-β in WT MEF but not in the null MEF (Fig. 6A). Response of Cspg2 (Fig. 6B) expression was similar to Postn, whereas that of Fbln2 (Fig. 6C) was not much different between W10 and N47 cell lines, and an increase in N47 was seen at 12 h after treatment. Therefore, the expression changes observed in Postn and Cspg2 in the WT and menin-null MEFs may reflect the TGF-β–induced, menin-mediated expression changes.

**Consequence of Gene Expression Changes on Menin Loss**

Two other studies have looked at the expression changes in mouse in WT and menin-null conditions in embryos or fibroblasts using Affymetrix arrays. Four genes (Hoxc6, Cyt19, Hoxc8, and Asb4) with reduced expression (1.29- to 1.56-fold) in menin-null embryos were identified (6). None of these appeared in our list, perhaps because of differences between whole embryos and MEFs. Menin-null MEFs, immortalized with retrovirus expressing human papillomaviral E6 and E7, and infected with retrovirus expressing menin, led to the identification of 25 transcripts each with >2-fold down (18) and up (12). Of these, Sdfr2 and Lgals3 were also found to...
be in our list of underexpressed and overexpressed transcripts, respectively, in menin-null MEFs. Differences in the transcripts printed on different array platforms, the methods in immortalization of MEFs may contribute toward an incomplete overlap of the transcripts observed. Our posting of the expression analysis on the entire set of 21,000 transcripts provides an opportunity for other investigators to compare their results with these data. 7

The menin-null cell lines were found to respond poorly to TGF-β. This is consistent with earlier observations that blocking of menin function by antisense oligos results in blocking of TGF-β–mediated activation of a Smad3-responsive promoter (8). As discussed below, some of the known TGF-β/Smad3–induced target genes (19) in dermal fibroblasts were underexpressed in menin-null MEFs that included Iγfbp3 and Nid1 and members of the collagen family (Col1a2, Col3a1, Col5a2, and Col6a1). In addition, Fbln2, Postn, and Cspg2 were also underexpressed in menin-null MEFs, are required for (or markers of) heart development, and are induced by TGF-β. Unlike in a menin-null MEF, expression of Cspg2 and Postn increased in a WT MEF on TGF-β treatment (Fig. 6), supporting that menin might play a role in TGF-β–mediated expression changes of some of the extracellular matrix genes.

Menin-null embryos display defects in neural tube, heart, skeletal, and liver development (15, 17). It is apparent from transcript profiling of embryonic fibroblasts that many genes affected by the loss of menin are important for extracellular matrix/space and cell adhesion. Among these genes, Fbn2...
and Postn display the highest degree of down-regulation between menin-null and WT fibroblasts. Both Fbln2 and Postn are prominently expressed in the endocardial cushion tissue within the developing heart tube during embryonic days 10 to 13 (20, 21). In addition, both genes are expressed during skeletal development (22). Another extracellular matrix protein whose expression was similarly down-regulated in the menin-null fibroblasts is versican (Cspg2), a large proteoglycan that is critical for endocardial cushion tissue formation during cardiac development (23). The endocardial cushion tissue, which ultimately divides the straight heart tube into four chambers, is formed by the process of epithelial-mesenchymal transformation in response to signaling of the TGF-β and bone morphogenetic protein growth factor families (24). We suggest that the reduced expression of Fbln2, Postn, and Cspg2 in the absence of menin could contribute to the defects in heart morphogenesis of menin-null embryos.

**Materials and Methods**

**Establishment of MEF Cell Lines**

The embryos were dissected and treated with trypsin (5 min at 37°C) to dissociate them into a single-cell suspension. The cell suspensions were plated onto gelatin-coated six-well plates with DMEM + 15% fetal bovine serum. The cells were allowed to immortalize and transferred to larger plates as needed. Genotypes were established by PCR of amnion DNA from embryo using primers as described earlier (25). Expression of Men1 was analyzed by RT-PCR, and reverse-transcribed RNA was used
with primers in exons 2 and 6: TGCCCGATTCCCGCTCAGA (691F) and GCGCCATGGGGTATCTTTCC (1269R).

Western Blot Analysis

Equal amounts of whole-cell lysates (10-20 μg) were separated on a 12% Tris-glycine gels and transferred to nitrocellulose membranes. Menin antibodies were elicited in rabbits using the COOH-terminal peptide (4). Rabbit anti-Hsp25 (Hspb1; Stressgen Biotechnologies Corp.), monoclonal p84 (GeneTex, Inc.), and goat anti-rabbit and anti-mouse secondary (IgG-horseradish peroxidase) antibodies (KPL, Inc.) were from commercial sources. The membranes were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Inc.).

For determination of secreted proteins, 100 μL of serum-free culture medium were precipitated with 900 μL of 100% ethanol for 1 h at −70°C, run on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen Corp.), and subjected to Western blotting using the ECL Plus detection reagents (GE Healthcare). Primary antibodies used were polyclonal antibodies against mouse OSF-2/Postn (R&D Systems).

Spectral Karyotyping

WT (W10, W46, and W50) and menin-null (N14, N46, N47, and N41.4) cell lines were analyzed for chromosomal changes using spectral karyotyping as described earlier (26).

Soft Agar Assay

Soft agar colony-forming assays were done in duplicate as described previously (27). Cells were trypsinized, and 5,000 to 6,000 cells were resuspended in 3 mL of molten 0.33% agar and poured over a 5-mL 0.66% solidified layer of agar in 6-cm plates. The agar solutions were made in complete DMEM. Agar plates were incubated at 37°C in a 5% CO₂, humidified incubator and observed after 2 weeks for colony formation.

Luciferase Assays

WT and menin-null MEF cell lines were plated in six-well plates at a density of 1 × 10⁵ per well. One microgram of the reporter construct (3TP-Lux, provided by the late Dr. Anita Roberts, National Cancer Institute, Bethesda, MD) was transfected using LipofectAMINE 2000 (Invitrogen). For evaluation of the effect of menin, 0.5 and 1 μg of either Men1 expression construct (pCMV-sport-Men1) or the vector (pCMV-sport) alone were cotransfected with 3TP-Lux construct. After 24 h, the medium was replaced with low-serum (0.2% fetal bovine serum) medium to reduce the amount of TGF-β that may be present in the fetal bovine serum. Ten hours later, 2.5 ng/mL TGF-β (R&D Systems) was added to the medium, and the next day, the cells were lysed. The reporter constructs were cotransfected with 1 ng of Renilla luciferase construct as an internal control. Luciferase levels

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FIGURE 4. Verification of expression changes at the RNA level by Northern blot (A) and at protein level by Western blotting (B and C) and flow sorting (D). Total RNAs from each MEF cell line were run on agarose gels and transferred to membrane and hybridized with labeled full-length cDNA probes for Fbln2, Postn, and Hspb1. Menin-null MEFs generally show decreased expression of Fbln2 and Postn and increased expression of Hspb1 compared with WT. B. Increased expression of Hspb1 protein in menin-null cells is also verified by Western blot analysis. C. Western blot analysis of cell culture supernatants shows decreased expression of Postn protein in menin-null MEFs. An extracellular matrix protein antibody that can serve as a loading control is not available; however, same amount of proteins was loaded in each lane. D. Flow cytometric analysis of Cd24a expression in WT (W10, W26, W46, and W50) and menin-null (N12, N17, N40, N14, N47, and N41.4) MEFs. With the exception of W10, WT MEFs showed lower Cd24a expression compared with menin-null MEFs.
were measured using the Berthold Centro LB 960 luminometer and reagents from the Dual-Luciferase Assay kit (Promega Corp.). The enzyme activity was normalized relative to Renilla luciferase activity.

**cDNA Microarrays**

The cDNA microarrays consisted of 21,120 cDNA clones that included 15,000 from embryonic cDNA libraries (28) representing 16,219 unique mouse UniGene clusters. cDNA microarrays were fabricated in the National Human Genome Research Institute microarray core facility, and one print batch of 100 slides was reserved for this study. MEF cell lines were grown to 70% confluence and harvested, and total RNA was extracted using Trizol reagent (Invitrogen) and RNeasy Maxi kit (Qiagen, Inc.). RNA was reverse transcribed, labeled by incorporation of Cy5 or Cy3 fluorochrome, and hybridized to the slides using standard protocols. For each menin-null MEF cell line (N12, N17, N40, and N47), eight two-channel hybridizations were done against all four WT MEF cell lines (W10, W26, W46, and W50) with standard fluorescent dye swapping protocol, obtaining a total of 32 expression profiles. For further comparison, two pooled RNAs (menin-null and WT pairs) were flipped for dye swap hybridization, we chose single-group t test for significant differential expression with all 32 hybridizations in one group. The t test was done using Avadis 4.0 (Strand Genomics), with asymptotic P value and Benjamini-Hochberg correction for multiple tests.

**Northern Blot Analysis**

Total RNA (20 μg) was fractionated on 1.2% agarose-formaldehyde gel, transferred to nylon membrane, cross-linked using UV, and hybridized using ExpressHyb (Clontech) with 32P-labeled cDNA probes. The full-length cDNA inserts were isolated by NotI and MluI digestion of the plasmids: IMAGE clone IDs 3490759, 4457222, and 3498180 had cDNA inserts for Fbln2, Postn, and Hspb1/Hsp25, with insert sizes of 3,969, 3,109, and 862 bp, respectively.

**Cd24a Expression by Flow Cytometry**

Trypsinized cells (3 × 10⁶) were washed with PBS twice and suspended in 150 μL bovine serum albumin (1% in PBS) solution. Aliquots (50 μL, 1 million cells each) were treated for 1 h with 2 μL FITC-Cd24a or FITC-isotype antibodies (eBioscience), and the third aliquot was used as untreated control. Cells were washed and then suspended in 400 μL of bovine serum albumin solution. A FACSCalibur and CellQuest

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**FIGURE 5.** Quantitative RT-PCR using Taqman assay with RNA from WT and menin-null MEFs in pooled (A) and individual (B and C) MEFs. RNAs from four individual clones were pooled for both WT (WT POOL) and menin-null (NULL POOL) MEFs. Relative expression was calculated by the 2⁻ΔΔCT method using the expression of β2-microglobulin as internal control. A, Expression for 12 transcripts in WT and the menin-null MEFs. Bottom, the expression data are also represented as fold change in menin-nulls relative to WT MEFs. Black columns, WT MEF pool samples; gray columns, menin-null MEF pool samples. In agreement with array data, Cspg2, Igfbp3, Wnt5a, Pbx3, Shhkbp3, Sgk, and Cont2 are noted to be higher and Btg2, Peg3, Cp, Vil2, and Clu are lower, respectively, in WT MEFs. B and C, Relative expression in four WT (W10, W26, W46, and W50) and four menin-null (N12, N17, N40, and N47) individual MEF cell lines is shown for transcripts Mox2, Cd24a, Btg2, and Cspg2.
software (Becton Dickinson) was used to collect and analyze the data.

**Quantitative RT-PCR**

Quantitative PCR was carried out using the Taqman assay. cDNA was prepared using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and an oligo(dT) primer. cDNA template and Taqman probes (50 ng) were used for ABI PRISM 7700 (Applied Biosystems). Taqman probes were purchased from Applied Biosystems: Btg2 (Mm00476162_m1), wnt5a (Mm00437347_m1), Pbx3 (Mm00479413_m1), Vill2 (Mm00447761_m1), Peg3 (Mm00493299_m1), Men1 (Mm00484963_m1), Sbhkbp1 (Mm00451715_m1), Ccnb2 (Mm00432351_m1), Cp (Mm00432654_m1), Sgk (Mm00441380_m1), Csgp2 (Mm00490179_m1), Igfbp3 (Mm00515156_m1), Cd24a (Mm00782538_sH), Max2 (Mm00487740_m1), Clu (Mm00437347_m1), Postn (Mm01289595_m1), and Fbln2 (Mm00118873_m1). β2-microglobulin (Mm00437762_m1) or Gapdh (Mm00468022_m1) was used as internal control. The relative mRNA expression level was calculated using the comparative expression level \(2^{-\Delta \Delta C_T}\) method (CT is the threshold cycle; ref. 30). The

**FIGURE 6.** Expression of Postn (A), Csgp2 (B), and Fbln2 (C) on TGF-β treatment of WT and menin-null MEFs. RT-PCR, quantitative RT-PCR (RT-qPCR), and Western blot analysis of a WT (W10) and a menin-null (N47) MEF cell line treated with different concentrations (0, 2.5, 5, and 10 ng/mL) of TGF-β for varying lengths of time (12, 24, and 48 h). RT-PCR: the ethidium bromide gels show the expression by RT-PCR; Gapdh is used as control to show that the same amount of template is used in the reaction. Quantitative RT-PCR: the quantitative expression was calculated by the \(2^{-\Delta \Delta C_T}\) method using the expression of Gapdh as internal control; the data are presented to show the fold change in expression of TGF-β–treated samples compared with the untreated, represented as 1. A. RT-PCR, quantitative RT-PCR, and Western blot analysis indicate increased expression of Postn in response to TGF-β treatment of WT MEF. A similar response was seen for Csgp2 (B) but not for Fbln2 (C) expression.
experiments were repeated in triplicates, and the mean value with SD is reported.

**TGF-β Treatment and RT-PCR**

WT (W10) and menin-null (N47) MEF cell lines were plated and replaced with low-serum (0.2% fetal bovine serum) medium after 24 h, and 12 h later, varying amounts of TGF-β were added to the medium (0, 2.5, 5.0, and 10.0 ng/mL), and cells were harvested 12, 24, and 48 h after treatment. Isolation of total RNA and reverse transcription were as described above. RT-PCR was done using primers for *Postn* (GCTCCTGTAAGAACCAGGG and CCCCCCCCCTCATATA), Cspg2 (ACCAGCAGGTGACCTTGAAC and ACAACATCAGTGGTTCTGGGAC), Fbn3 (GAACTTCTCGGATGCTAGG and CAACTGGCAGGGTGTCTAG), and Gapdh (CAGTGGCAGAATGGGAGATTTGCCGAGTG and AATTTGCCGTGAGTGGAGTC).

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**References**

Molecular Cancer Research

Mouse Embryo Fibroblasts Lacking the Tumor Suppressor Menin Show Altered Expression of Extracellular Matrix Protein Genes

Youngmi Ji, Nijaguna B. Prasad, Elizabeth A. Novotny, et al.


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