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Menin induces endodermal differentiation in aggregated P19 stem cells by modulating the retinoic acid receptors

Jyotshnabala Kanungo^{1,*} and Settara C. Chandrasekharappa¹

¹Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD 20892

^{*}Division of Neurotoxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

Abstract

Menin, a ubiquitously expressed protein, is the product of the multiple endocrine neoplasia type I (*Men1*) gene, mutations of which cause tumors primarily of the parathyroid, endocrine pancreas, and anterior pituitary. Menin-null mice display early embryonic lethality, and thus imply a critical role for menin in early development. Here, using the P19 embryonic carcinoma stem cells we studied menin's role in cell differentiation. Menin expression is induced in P19 cell aggregates by retinoic acid (RA). Menin over-expressing stable clones proliferated in a significantly reduced rate compared to the empty vector harboring cells. In aggregates, specific populations of menin over-expressing cells displayed the characteristic of an endodermal phenotype by the acquisition of cytokeratin Endo A expression (TROMA-1), a marker for the primitive endoderm, with a concomitant loss of the stem cell marker SSEA-1. Menin's ability to induce endodermal differentiation in specific populations of the aggregated cells in the absence of RA implied that menin could substitute RA by inducing a set of target genes that are RA-responsive. Menin over-expressing cells upon aggregation showed a robust expression of retinoic acid receptors (RAR), RAR α , β and γ compared to the empty vector harboring cells. Moreover, endodermal differentiation was inhibited by the pan-RAR antagonist Ro41-5253, suggesting that menin could induce endodermal differentiation of uncommitted cells by functionally modulating the RARs.

Keywords

Menin; endodermal differentiation; stem cells; retinoic acid receptor; cytokeratin EndoA

Introduction

Menin, a 610-amino acid nuclear protein, is encoded by the multiple endocrine neoplasia type I (*Men1*) gene and mutations in *Men1* cause an autosomal dominant syndrome characterized by tumors of the parathyroid, endocrine pancreas, anterior pituitary and other tissues [1]. Menin is ubiquitously expressed, and found in a variety of human and rodent cell lines and tissues [2–5]. The amino acid sequence of menin does not show homology to any known functional domains, but is known to bind JunD thereby regulating cell growth [6, 7].

Address correspondence to: Jyotshna Kanungo, Ph.D, Division of Neurotoxicology, National Center for Toxicological Research, FDA, 3900 NCTR Road, Bldg 53D, Rm 203O, Jefferson, AR 72079, Tel: 870-543-7591, Fax: 870-543-7745, jyotshnabala.kanungo@fda.hhs.gov. Settara C. Chandrasekharappa, PhD, Cancer Genetics Branch, National Human Genome Research Institute, NIH, 50 South Dr, Bldg 50, Room 5232, Bethesda, MD 20892, Tel: 301-402-2344, Fax: 301-480-1241, chandra@mail.nih.gov.

In addition to being a tumor suppressor, many other cellular functions of menin have been reported. Menin-null mouse embryonic fibroblasts (MEF) enter senescence earlier than their wild-type counterparts [8], and are deficient in PPAR γ -mediated adipocyte differentiation [9]. Menin-null ES cells exhibit a deficiency in hematopoietic differentiation [10]. While the role of menin in spermatogenesis [11], and duct cell differentiation in mouse submandibular gland [12] has been implicated, its requirement for both early differentiation of osteoblasts, but inhibition of their later differentiation, has been established [13, 14]. Menin mediates its effects on early osteoblastic differentiation by interacting with Smads and Runx2 [14], and suppresses osteoblast differentiation by antagonizing the AP-1 factor and JunD [15]. Thus, menin expression modulates mesenchymal cell commitment to the myogenic and osteogenic lineages [16]. Furthermore, Menin plays a critical role as a component of HMTase complex in transcriptional activation *via* H3K4 methylation [17, 18]. Menin influences *Hoxa9* expression and thereby regulates hematopoiesis and myeloid transformation [19–21], and is required for MLL-associated leukemogenesis [22]. Homozygous loss of menin in mice results in early embryonic lethality illustrating the importance of menin in early development [12, 23]. In order to elucidate the role of menin during early developmental processes, we sought to use the P19 cells, where we could experimentally modulate menin expression levels, so as to correlate with the cell behavior in terms of its growth and differentiation.

The P19 embryonic carcinoma stem cells are derivatives of the inner cell mass of a mouse blastoderm and are multipotent cells capable of giving rise to all three germ layers [24]. These cells are anchorage-independent, do not display contact inhibition, and are tumorigenic [25]. P19 stem cells respond to a number of morphogens and thus can differentiate into primitive endoderm, mesoderm, and ectoderm as well as to neuron-like cells [26] and beating cardiomyocytes [27, 28]. The P19 cells, therefore, serve as an ideal model system for studying early embryonic development and differentiation. Treatment of P19 cells in monolayer with low concentration (10 nM) of retinoic acid (RA) leads the cells to differentiate into primitive endoderm-like cells whereas treatment of cell aggregates with high concentrations of RA results in their differentiation into neurons and glia [27, 29, 30]. RA-induced endodermal differentiation of P19 cells requires G α 13 and G α 12 [31–33]. Intracellular signaling pathways involved in this process include the JNK-signaling cascade linking other members of the pathway such as MEKK-1, MEKK-4, and MKK-4 [31–34]. A later study demonstrated that JLP (JNK-interacting leucine zipper protein), a scaffold protein is critical in the retinoic acid-induced endodermal differentiation in P19 cells [35].

In the current study, we explored the role of menin in differentiation of P19 stem cells. P19 cells express menin and its expression is upregulated in cell aggregates by addition of RA (10 nM or more). Menin over-expressing stable clones grew in a significantly slower rate compared to their empty-vector control counterparts. Men1 over-expression was sufficient to induce endodermal differentiation of some of the aggregated cells in the absence of RA. These cells in monolayers however did not show any endodermal phenotype with or without the treatment of 10 nM RA. These results implied a complex role of menin that is dependent on the spatial status of the cell and many other additional factors, such as, cell adhesion that can modulate its function. Menin induced robust increase in expression of the RA receptors, RAR α , β and γ at the transcriptional level in aggregated cells but not in cells monolayers. Furthermore, the pan-RAR antagonist Ro41–5253 inhibited menin-induced endodermal differentiation of the aggregated cells. These results suggest that RA-independent endodermal differentiation of P19 aggregates requires menin. Although RA enhances menin expression in P19 cell aggregates, menin by itself can regulate the expression of all the three RARs, thus seemingly providing the potential ingredient for cellular differentiation.

Materials and methods

Cell culture

The P19 embryonic carcinoma stem cells were purchased from the American Type Culture Collection (Manassas, VA). Both the stable transfectants and the wild-type clones were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified atmosphere of 6% CO₂.

Antibodies and reagents

A rabbit polyclonal antibody raised against the C-terminal peptide of human menin (anti-menin) has been described earlier [36]. TROMA-1, a rat monoclonal antibody against cytokeratin Endo A, and a mouse monoclonal antibody that recognizes the stem cell-specific cell surface antigen (SSEA-1), were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). A mouse monoclonal anti-p84 antibody was purchased from GeneTex, Inc, (USA). All trans-retinoic acid was purchased from Sigma (USA), and Ro41-5253, the pan-RAR antagonist was purchased from Biomol International, PA (USA). Neomycin (G418) and Puromycin were purchased from Invitrogen (USA) and Sigma (USA), respectively.

Plasmids and stable transfection

pCDNA3 (empty vector) and pCDNA3-Men1 plasmid [7] were used to transfect P19 cells and following neomycin selection, three stable clones each were isolated. Neomycin (G418) selection was with 400 µg/ml concentration and stable clones were maintained in 100 µg/ml concentration

Primers

Human *Men1*: 5'-GGTGAAGAAGGTCTCCGATG-3' (forward), 5'-ACACTACCCAGGCATGATCC-3' (reverse). Mouse *Men1*: 5'-TCGTCGACAACGGCTCCGGC-3' (forward), 5'-CCAGCCAGGTCCAGACGCAG-3' (reverse). Mouse *RARα*: 5'-ACGAACAACAGCTCAGAAC-3' (forward), 5'-TGCAGATTTCGAGAATCAG-3' (reverse). Mouse *RARβ*: 5'-ATACCCAGAGCAAGACAC-3' (forward), 5'-CCACAGATTAAACAGATGGCAC-3' (reverse). Mouse *RARγ*: 5'-GACGCAGCATTCAGAAAAAC-3' (forward), 5'-AGCCCTCCTCTTTTACCTCC-3' (reverse). Mouse *β-actin*: 5'-TCGTCGACAACGGCTCCGGC-3' (forward), 5'-CCAGCCAGGTCCAGACGCAG-3' (reverse). Mouse *GAPDH*: 5'-GGTCCTCAGTGTAGCCCAAG-3' (forward), 5'-AATGTGTCCGTCGTGGATCT-3' (reverse).

RNA isolation and RT-PCR

Total RNA was extracted using phenol-chloroform (TRIzol reagent; Invitrogen). cDNA was prepared using the First Strand Synthesis kit (Roche, Indianapolis, IN, USA). The experiments were repeated three times.

Cell proliferation assay

P19 stem cell proliferation assays were performed using the MTT Cell Proliferation Assay Kit (Roche, USA). For proliferation assays, P19 cells were washed three times in DMEM/FCS, and 1×10^5 cells were cultured in P19 growth medium for up to 4 days. Triplicate wells for each time point were established at time zero. For EV and Men1-overexpressing clones, 12-well plates were used. At 0, 24, 48, 72 and 96 hrs, 200 µl of Dye solution was added and the plates were incubated for 4 hours in the incubator. Then 2 ml of the Solubilization/Stop mix was added and the plates were incubated in the incubator overnight.

The contents were mixed using a Pasteur pipet to obtain a uniform color. Two hundred microliters of the contents were transferred to the 96-well plate and absorbance (OD) was measured. The ODs were converted to cell numbers based on a Standard curve that was obtained by using a known number of cells for the assays.

Immunoblotting

Total cell lysates were subjected to electrophoresis in SDS on 4–20% polyacrylamide gels. The resolved proteins were transferred electrophoretically to nitrocellulose blots. The blots were stained with primary antibodies (anti-menin or anti-p84), and the immune complexes were made visible by the chemiluminescence kit (Pierce, USA) in tandem with a second antibody coupled with horseradish peroxidase (Santa Cruz Biotechnology, Inc., CA, USA).

Induction of P19 stem cell differentiation

P19 cells cultured as monolayers on tissue culture plates in the Dulbecco's modified Eagle's medium (Invitrogen, USA) with 10% fetal bovine serum (Invitrogen, USA) were induced to primitive endoderm by the addition of 10 nM all-trans retinoic acid (RA) 4 days and then cultured for two additional days without RA. To promote cellular differentiation in cell aggregates, cells (7×10^4 /ml culture media) were cultured in normal growth media supplemented with 10 nM RA for 4 days in bacterial Petri dishes. After 4 days, floating aggregates were harvested, dissociated by treatment with phosphate-buffered saline (PBS) containing 1 mM EDTA, and then transferred to tissue culture plates and cultured in medium lacking RA for an additional two days [32].

Indirect immunofluorescence assay

A rabbit polyclonal antibody against human menin was used (1:500 dilution) to detect menin. The staining of the embryonic antigen SSEA-1 with mAb MC-480 and the endoderm-specific marker antigen cytokeratin Endo A by the monoclonal antibody TROMA was performed with antibodies purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IO). P19 cells were cultured, stained, and subjected to analysis by epifluorescence microscopy as described previously [33, 37].

Results

Retinoic acid induces menin expression in P19 stem cell aggregates

P19 cells, when cultured in monolayers in presence of 10 nM RA, differentiate into primitive endoderm-like cells. When P19 cell aggregates are treated with high concentrations (100 nM or more) of RA, neuronal differentiation occurs. Whether during the course of this event, menin expression level changes, was our primary question in order to explore a link between menin and P19 cell differentiation. P19 cells cultured in bacterial Petri dishes, used to allow the cells to aggregate and form embryoid bodies, were treated with RA (10 nM or 100 nM) for up to three days. The control sets of cells were treated with ethanol (vehicle). Immunoblot analyses of the extracts prepared from these cells revealed an induction of menin expression from day one in RA-treated cells compared to the control cells (Fig. 1A, B). Initial experiments showed that there was no difference in the outcome when 10 or 100 nM RA doses were used (data not shown). Therefore, we performed the rest of our experiments using the 10 nM dose. Although there was an increase in menin expression in the control cells on day 2–3, a significant increase of menin level in RA-treated cells was apparent in these time periods (Fig. 1A, B).

The subsequent question was to determine, if the increase in menin level in RA-treated cells is a consequence of transcriptional regulation of Men1 by RA. In order to address this, we performed semi-quantitative RT-PCR, which demonstrated that, in fact, Men1 is

upregulated, although to a very modest level, by RA, beginning day 1 (Fig. 1C). The results also show that aggregation itself induces menin expression to a lesser extent than that by RA treatment (Fig. 1C). These results suggested that menin expression is responsive to RA further directing emphasis on two aspects, first, Men1 could be a RA-responsive gene, and second, menin may play a role in P19 cell differentiation.

Men1 over-expression attenuates cell growth

Based on the observation that RA induces menin expression in the P19 stem cell aggregates, we explored whether menin itself could induce cellular differentiation in the absence of RA. To this end, we established several stable clones that expressed human Men1 and the empty vector (pCDNA3). For comparison we selected two stable clones from each. Semi-quantitative RT-PCR analyses demonstrated that the clones over-expressing Men1 were positive for human Men1 while the empty vector clones did not express human Men1 (Fig. 2A). Immunoblot analyses, using a polyclonal antibody that recognizes both the murine and human menin, further confirmed a modest but significant increase in the menin expression level in the Men1 over-expressing clones over that in the empty vector clones (Fig. 2B, C). Immunostaining of these cells using the menin antibody revealed an increase in the nuclear menin content in the Men1 over-expressing clones compared to the empty vector clones (Fig. 2D).

We then explored whether menin's growth suppressive activity was functional in these cells. Analyses of cell proliferation rate over a period of 0–96 h, using an MTT Assay Kit, demonstrated a significant reduction in cell growth in Men1 over-expressing cells compared to the cells harboring the empty vector (Fig. 2E). These results show that P19 cells are responsive to menin's growth suppressive action.

Menin induces endodermal differentiation in cells aggregated in the absence of retinoic acid

In order to examine the differentiation potential of the Men1 over-expressing clones, we performed differentiation induction cultures that span 6 days, four days as cell aggregates in the presence or absence of RA followed by a two-day culture in tissue culture dishes. Two clones each for the Men1 over-expressing and empty vector-harboring cells were used for these experiments. It is important to note that Men1 over-expressing cells although made embryoid bodies when allowed to aggregate, could not survive the RA treatment. These cells began to die from the very first day (Fig. 3A). Since our goal was to study the role of menin in endodermal differentiation, we continued the experiments in the absence of RA. As expected, the empty vector clones were only positive for SSEA-1 antigen in the absence of RA, but were negative for TROMA-1 (Fig. 3B, upper panel). In contrast, Men1-overexpressing cells showed an endodermal phenotype with acquisition of TROMA-1 cross-reactivity and loss of SSEA-1, indicating that Men1 over-expression was sufficient to direct the aggregated cells to differentiate into an endodermal phenotype (Fig. 3B, lower panel). The increased expression of TROMA-1 in Men1 over-expression clones was apparent in Western blots with TROMA-1 antibodies (Fig. 3C, D).

Menin induces RAR gene expression during cell aggregation but not in cells cultured in monolayers

Menin being sufficient to induce endodermal differentiation in absence of RA implied that menin could replace RA by inducing the same pathway(s) RA is capable of inducing during the differentiation process. To address both the issues, we hypothesized that menin could regulate the expression of some or all the three RARs; or/and at the same time could be involved in their activation in a ligand-independent manner. Initially, cells were aggregated to begin the differentiation process. Two days post-aggregation, the cells were collected and

total RNA was isolated for cDNA synthesis and subsequent RT-PCR analyses. Cells were also processed for nuclear extract preparation in order to be used for immunoblot analyses. First, semi-quantitative RT-PCR analyses revealed that there was a significant increase in RAR α , β and γ expression in the Men1 over-expressing cells compared to the empty vector-harboring cells (Fig. 4A). However, menin over-expressing cells when cultured in monolayers did not show an upregulation of the RAR α , β and γ expression (Fig. 4B). These results suggest that menin-induced upregulation of the RARs could be a primer for differentiation and their subsequent activation could cause cell differentiation.

Menin-induced endodermal differentiation is abolished by the pan-RAR antagonist Ro41-5253

In order to examine whether menin induced endodermal differentiation by inducing the RARs and potentially activating them, we used the pan-RAR antagonist, Ro41-5253. During the first four days of RA treatment of the cell aggregates, we included 10 μ M of Ro41-5253 or DMSO as control, in the culture media. Six days post-differentiation process, cells were immunostained to monitor the expression of SSEA-1 and TROMA-1. Men1 over-expressing clones differentiated into the endodermal phenotype, but failed to do so in the presence of Ro41-5253 (Fig. 4C). These results suggested that menin's ability to induce endodermal differentiation in the aggregated P19 stem cells is dependent on the RARs.

Discussion

Menin is a nuclear protein that has tumor-suppressive activity [23, 36]. Menin null mice are embryonically lethal [8, 23]. Therefore, using conditional knockout mice, some of menin's cellular functions have been specifically depicted, e.g., its role in hematopoiesis [19]. These findings portray a multifaceted role of menin in cellular differentiation, few of which are revealed and more remains to be explored. We took advantage of the P19 stem cells which are pluripotent, respond to a variety of differentiating agents and differentiate into the three primitive germ layer-like lineages that are precursors to the beginning of early development [24, 26, 38]. When treated with 10 nM of RA, P19 cells in monolayers undergo endodermal differentiation [26, 27]. Our results show that P19 cells express endogenous menin. Upon aggregation, with RA treatment we observed an upregulation of menin that occurred at the transcriptional level, suggesting a remote possibility that menin might play a role in cellular differentiation. Of the known inducers of endodermal differentiation in P19 cells, G α 12 and G α 13 [31–33] and the JNK scaffolding protein (JLP) are known to mediate their cellular signaling via JNK [35]. No nuclear protein that is involved in P19 endodermal differentiation has been reported yet, although the myocyte enhancer factor 2C (MEF2C) and NkX2-5 have been shown to be sufficient to induce mesodermal differentiation and cardiomyogenesis in P19 cells in the absence of differentiation-inducing agents [39]. That menin is a tumor suppressor is further validated by our growth rate analysis results in P19 cells, which suggest that the potential pathway(s) operating downstream of menin to attenuate cell growth is intact in these cells.

We speculate a primordial function of menin during very early development when embryonic pattern formation occurs, and the ectoderm, endoderm and mesoderm lineages are established. However, menin over-expressing cells in monolayers were resistant to RA induction of endodermal differentiation (data not shown). These cells lost the stem cell marker, SSEA-1. In these cells, RA did not induce the expression of the RARs indicating that due to the loss of stem-ness, these cells lost the ability to respond to the RA signals.

In a complementary experiment, we discovered that Men1 over-expression did not induce endodermal differentiation in monolayer cultures (data not shown), but did so upon cell aggregation, in the absence of RA. Previous studies have shown G α 12 and G α 13

suppressing P19 cell growth [40]. However Gα12- and Gα13-overexpressing cells undergoing endodermal differentiation in monolayers [31–33] stands in contrast to our finding. Nonetheless, these collective findings do point toward the obvious differential pathways that might be operating when the cell membrane-localized G-proteins and nuclear menin bring about an identical biological readout.

Although the downstream pathways involving MEKK-MEK-JNK have been dissected that link Gα12 and Gα13 to endodermal differentiation in P19 cells [34, 37], a downstream event emanating from a nuclear protein like menin, did require further clues so as to connect menin to endodermal differentiation. In this regard, we contemplated on the substitutive function of menin for RA, and also the importance of the requirement of cell aggregation for menin to induce endodermal differentiation. RA is the bioactive derivative of dietary Vitamin A and β-carotene, which is known as a signaling molecule controlling cell proliferation and differentiation [41]. Menin being sufficient to induce the transcriptional upregulation of the three RARs again reinforces the notion that although menin is an RA-responsive gene, it may also act as a transcriptional coactivator at the promoters of the three RARs. Menin not only enhances RARs' expression, but also induces endodermal differentiation that RA is capable of doing by ligand-dependent activation of its RARs. These results imply that menin could also activate the RARs thereby playing a role in regulating the RA-responsive gene transcription, of which, one or more could act as effectors for the endodermal differentiation.

Spontaneous expression of RARβ2 has been shown during DMSO-induced mesodermal differentiation in P19 cells [42]. Nuclear transcription factors can induce P19 cell differentiation in the absence of the inducers like RA and DMSO. Neurogenin1, Sox6 and Stra13 over-expressions have been shown to be sufficient to induce neuronal differentiation of P19 cells in the absence of retinoic acid [43–45]. In the absence of DMSO, transcription factors that are capable of inducing mesodermal differentiation upon their over-expression in P19 cells include MEF2C and Nkx2.5 [39], GATA-4 [46], MyoD [47] and b-catenin [48]. However, while 1.5–3% of MEF2C expressing cells become cardiomyocytes, 30–60% of cells over-expressing Nkx2–5 differentiate in to cardiac muscle cells [39]. In our case, we found ~ 10–20% cells over-expressing menin displayed endodermal phenotype. These results suggest that a minor population of cells differentiating into the endodermal phenotype may be due to their location in the embryoid body. The inner mass of cells of the embryoid bodies are in a complete cell-cell contact environment, whereas the outer layers of cells are partially in contact with other cells in the embryoid bodies. Either group of cells differentiating into the endodermal phenotype would not only justify the low percentage of differentiated cells observed in menin over-expressing cells, but also would emphasize on the importance of cell-cell adhesion contributing toward the differentiation process in synergy with menin.

Our results imply that menin could regulate cellular differentiation in a manner that is spatially (on the basis of cell microenvironment, cell adhesion, inter-cellular signaling, etc) determined. In addition, menin's interaction with other players in these biological processes seems obvious. In summary, we show that RA modulates menin expression in P19 cell aggregates. We also provide evidence that menin is sufficient to induce endodermal differentiation in presence of cell aggregation and the differentiation is inhibited by the pan-RAR antagonist Ro41–5253. Finally, we demonstrate that menin itself induces robust expression of the RARα β and γ in P19 aggregates in the absence of RA, which we believe is critical for cell differentiation. Whether menin regulates the RARs' transcriptional activation potential remains to be examined and so is the regulation of other downstream target genes that are critical for endodermal differentiation. In conclusion, the current study

presents evidence that menin is a key player in the RA signaling pathway and is critical for endodermal differentiation of aggregated pluripotent P19 stem cells.

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Figure 1.

Induction of menin expression by retinoic acid (RA). P19 stem cells were cultured in the presence or absence of 10 nM RA in bacterial culture grade Petri dishes for three days. The resulting cell aggregates (embryoid bodies, EB) were collected at defined time points. (A) Cell lysates prepared from these cells were analyzed (80 μ g total protein in each lane) for menin expression by immunoblotting using an anti-menin antibody. P84 protein expression serves as a control. (B) Densitometric analysis of the immunoblots is presented as ratio of menin to p84 expression in the Vector and Men1 clones in the presence and absence of RA. Student's t test was used to determine significance (*) set at $p < 0.05$. (C) Subsets of cells from these culture groups were used to isolate total RNA from which cDNAs were synthesized. Semi-quantitative RT-PCR analyses using these cDNAs as templates show endogenous Men1 expression. Expression of β -actin is shown as control

Figure 2.

Slower proliferation rate of P19 stem cells stably over-expressing human Men1. (A) RT-PCR analyses of cDNAs synthesized from the RNAs isolated from the empty vector-harboring P19 clones (Vector), and pCDNA3-Men1-harboring P19 clones (*Men1*) show human Men1 expression. GAPDH expression is shown as control. (B) Western blot analyses of cell lysates prepared from the empty vector-harboring P19 clones (Vector), and pCDNA3-Men1-harboring P19 clones (*Men1*) show menin expression. The anti-menin antibody recognizes both the murine and human menin. Expression of a ribosomal protein, p84, is shown to indicate equal loading. (C) Densitometric analysis of the immunoblots is presented as ratio of menin to p84 expression in the Vector and Men1 clones. Student's t test was used to determine significance (*) set at $p < 0.05$. (D) Immunocytochemical analyses using anti-menin antibody demonstrate menin expression in the Vector and Men1 over-expressing P19 clones (red). DAPI staining shows nuclei (blue). (E) MTT cell proliferation assays show the growth rate of the P19 Vector and Men1 clones. Three clones each were used for the assays. Multiple culture wells for each clone for each time point were established at time zero. Proliferation rate was monitored over a period of four days. Experiments were replicated three times. Error bars represent the standard deviation about the mean values

Figure 3. Endodermal differentiation of Men1-overexpressing aggregated P19 cells in an RA-independent manner. (A) P19 empty vector (pCDNA3) clones (Vector) and Men1 over-expressing clones (Men1) were cultured in the bacterial Petri dishes in the presence or absence of 10 nM of RA. Cells were monitored for a period of four days. Bright field photomicrographs of cells in culture show embryoid bodies and particulate dead cells. (B) P19 empty vector (pCDNA3) clones (Vector) and Men1 over-expressing clones (Men1) were cultured in the bacterial dish in the absence of RA for four days. Cells were then dissociated and cultured in tissue-culture grade chamber slides for two more days. SSEA-1 staining for the stem cell-specific antigen (red), TROMA-1 staining for the primitive endoderm (green) and DAPI staining for nuclei (blue) are shown. (C) Immunoblot of cell lysates of P19 Vector and Men1 clones following 6 days in culture (4 days in bacterial Petri dishes and two days in tissue culture-grade Petri dishes) shows TROMA-1 expression. Expression of a ribosomal protein, p84, is shown as loading control. (D) Densitometric analyses of the immunoblots show the ratio of p84 to TROMA-1 expression. Student's t test was used to determine significance (*) set at $p < 0.05$

Figure 4. Induction of RAR gene expression in P19 cell aggregates over-expressing menin and inhibition of menin-induced endodermal differentiation by the pan-RAR antagonist, Ro41-5253. (A) RT-PCR analyses show RAR α , β and γ mRNA expression in cell aggregates of Vector and Men1 clones cultured for two days in the bacterial Petri dishes. GAPDH expression is shown as control. (B) RT-PCR analyses show RAR α , β and γ mRNA expression in Vector and Men1 clones cultured in monolayers. GAPDH expression is shown as control. (C) P19 Vector and Men1 clones were cultured in bacterial Petri dishes for 4 days in the presence of DMSO (vehicle) only or 10 μ M Ro41-5253. Cells were then dissociated and cultured in chamber slides for two more days. Indirect immunofluorescence was

performed using the stem cell marker SSEA-1 or the endodermal marker TROMA-1. Representative pictures show SSEA-1, TROMA-1 and DAPI (for nuclei) staining