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Reconstituted expression of menin in *Men1*-deficient mouse Leydig tumour cells induces cell cycle arrest and apoptosis

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ABSTRACT

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome caused by the inactivation of the responsible gene, *MEN1*. To date, the lack of *MEN1*-deficient cell lines derived directly from MEN1 tumours has hampered the detailed study of the *MEN1* gene. We have established several stable *Men1*-deficient Leydig cell tumour (LCT) lines derived from a Leydig cell tumour developed in a male heterozygous *Men1* mutant mouse. Our data show that these cell lines maintain the basic characteristics of Leydig cells in terms of both androgen synthesis and gene expression. Interestingly, reconstituted menin expression in one of *Men1*-deficient LCT cell lines resulted in cell growth inhibition, suggesting that the function of cell growth suppression of the menin pathway, apart from menin itself, is essentially preserved in these cells. Furthermore, we show that menin re-expression in these *Men1*-deficient cells leads to a block in the transition from G0/G1 to S phase of the cell cycle and an increase in apoptosis, accompanied by a marked increase of $p18^{INK4C}$ and $p27^{Kip1}$ expression. The current study therefore highlights the importance of menin expression in cell cycle and cell survival control in endocrine cells, and may provide insights into the mechanisms of tumour suppression by menin in related endocrine tumours.

1. Introduction

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant inherited disorder characterised by the occurrence of multiple hyperplasia and tumours of the parathyroids, endocrine pancreas, anterior pituitary, and adrenal cortex. In addition, other endocrine or non endocrine tumours can also be seen in MEN1 patients, such as carcinoid tumours, thyroid follicular adenomas, lipomas, angiofibromas and collagenomas.[1] The responsible gene for the syndrome, *MEN1*, is considered as a tumour suppressor, since its complete inactivation can often be evidenced by LOH (loss of heterozygosity) analysis in MEN1 tumours.[2,3]

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The *MEN1* gene encodes a 610 amino acid protein named menin,[4] which is mainly localised in the nucleus. Menin interacts with a number of proteins, incuding JunD,[5] several members of nuclear factor-κB,[6] Smad1, 3 and 5,[7,8] nm23,[9] RPA2,[10] FancD2,[11] ASK,[12] MLL1 and MLL2.[13–15] The fact that the majority of its protein partners are transcription factors and co-factors allows to hypothesise that menin could be involved in transcriptional regulation. Indeed, it has recently been reported that this protein may regulate the expression of several genes, including telomerase,[16] several hormones,[17,18] and the cyclin-dependant kinase inhibitors involved in cell cycle control, such as $p18^{INK4c}$ and $p27^{Kip1}$.[14,19,20] However, the *in vivo* relevance of the interactions between menin and its partners is so far poorly understood. The biological function of menin thus remains to be elucidated.

One of the difficulties in studying *MEN1* is the lack of *MEN1*-deficient cell lines derived directly from MEN1 tumours. This hampered greatly the detailed study of cellular and molecular effects of *Men1* gene expression. Nevertheless ectopic expression of menin represses proliferation and tumourigenesis of Ras-transformed NIH3T3 cells,[21] even though it is difficult to pinpoint the molecular mechanisms involved using such a cellular model, due to the presence of substantial levels of endogenous menin. Reduction of menin expression by transfected antisense cDNA in the rat duodenal crypt-like cell line, IEC-17, increased cell proliferation.[22] In addition, menin is critical for TGF-β induced inhibition of cell proliferation in pituitary tumour-derived cells.[8] Interestingly, Schnepp and colleagues have demonstrated that the reconstituted menin expression in *Men1*-deficient immortalised mouse embryonic fibroblasts (MEF) restored the sensitivity to apoptosis in response to UV and TNFα.[23] These findings suggest that menin regulates cell proliferation. However, it remains to demonstrate whether and how the cell proliferation inhibition function of menin exists in endocrine cells and whether it is disturbed in MEN1 tumours.

More recently, mouse knockout models for the *Men1* gene have been generated by several laboratories,[24–28] including ours. Interestingly, in addition to the endocrine tumours corresponding to those commonly described in MEN1 patients, we have observed the development of Leydig cell tumours with high frequency in our heterozygous *Men1* mice.[24] In order to carry out mechanistic studies on tumourigenesis related to *Men1* gene inactivation, we have attempted to establish stable *Men1*-deficient endocrine tumour lines derived from the tumours developed in heterozygous *Men1* mice. Here we report the successful establishment of several stable Leydig cell tumour (LCT) lines derived from a Leydig cell tumour developed in a male heterozygous *Men1* mutant mouse. All the established LCT lines have lost the wild-type *Men1* allele, and thus are the first *Men1*-deficient endocrine tumour lines so far reported. Most importantly, our data demonstrate that the reconstitution of menin expression exerts inhibitory effects on cell growth in one of these cell lines, through the mechanisms involving cell cycle blockage and apoptosis, as well as the increased expression of the inhibitory factors of cell cycle.

2. Materials and methods

2.1. Tumour isolation and primary culture

Testis tumours were excised from transgenic mice. The tumour capsule was gently removed, and the tumour cells were mechanically dissociated and pressed through a 40-μm Cell Strainer (Becton Dickinson Labware, Franklin Lakes, NJ). After one wash, they were plated in 12-well plates at about 10^6 cells per well, in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 25 mM glucose and supplemented with 15% (v/v) horse serum (Invitrogen), 2.5% (v/v) foetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 2 mM L-glutamine, and incubated in humidified air with 5% CO_2 at 37 °C. When cells reached about 50% confluency, they were transferred to 100-mm plates (Falcon) by trypsinisation with 0.05% (w/v) trypsin/0.5 mM EDTA. After about 3–5 passages, cells entered senescence and the medium was changed twice a week until cells restarted to grow (about 2 months in average). Then cells were divided twice a week and were cloned by limited dilution (one cell per well in 96-well), with the cloned lines being referred to as Leydig cell tumour (LCT) lines.

2.2. Cell lines and transfection assays

MA-10 (mouse Leydig cell line) cells were cultured in Waymouth's MB 752/1 medium (Life Technologies, Inc.) supplemented with 20 mM HEPES (GIBCO), 15% (v/v) horse serum, and 25 μg/ml gentamycin (GIBCO) at 37 °C with 5% CO_2. Mouse embryonic fibroblast (MEF) were grown in DMEM containing 25 mM glucose and supplemented with 10% (v/v) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 100 μM β-mercaptoethanol (Sigma) at 37 °C with 5% CO_2. Cells were transfected using lipofectamine 2000 (Invitrogen; Cergy Pontoise, France) according to the manufacturer's instructions.

2.3. Cytogenetic analyses

Metaphase spreads of cultured cells were prepared and stained by 4′,6′-diamidino-2-phenylindole (DAPI) according to the protocol previously described.[29]

2.4. Men1 mutant mice and genotyping

Mice carrying an inactivated *Men1* allele ($Men1^{+/T}$) were generated using a targeting vector, as described previously.[25] All animal experiments were conducted in accordance with accepted standards of human animal care and were approved by the International Agency for Research on Cancer's Animal Care and Use Committee. Southern blot analyses were performed to determine the presence of the wild type and targeted *Men1* alleles, by hybridising *Bam*H1 and *Bgl*II digested genomic DNA with a *Men1* gene probe, located immediately upstream of exon1.[25] The wild-type allele produced a 5.6 kb fragment, the targeted allele a 3.6 kb fragment. Genotyping by PCR was performed as described previously.[24]