Gene expression profiling in insulinomas of *Men1* β -cell mutant mice reveals early genetic and epigenetic events involved in pancreatic β -cell tumorigenesis

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Abstract

Mutations of the *MEN1* gene lead to the occurrence of multiple endocrine neoplasia type 1 (MEN1). To gain insights into the mechanisms of the tumorigenesis related to *MEN1* inactivation, we have used mice in which the *Men1* gene was specifically disrupted in pancreatic β -cells. In these mice, we observed full penetrance of insulinoma with defined histological characteristics of tumorigenesis. To identify the genetic factors taking part in the tumour development, we performed gene expression profiling analysis of these insulinomas at different stages. Here, we show that in late stage insulinomas, 56 genes are up-regulated and 194 are down-regulated more than fourfold compared with normal pancreatic islets. Clustering analysis reveals the deregulation of *Hox* gene family and the genes involved in cell proliferation and cell cycle control. The altered expression of *Igf2*, *Igfbp3* and *Igfbp6* as well as *cyclin A2*, *B2* and *D2* are confirmed by quantitative RT-PCR, with the overexpression of all the three cyclins found in early stage insulinomas. Moreover, an increased proportion of cyclin A2- and D2-expressing cells and the overexpression of insulin-like growth factor 2 (IGF2) protein are detected in mouse *Men1* insulinomas by immunostaining. Interestingly, the analysis of DNA methylation patterns by quantitative serial pyrosequencing reveals that four specific CpGs in the intragenic differentially methylated region 2 (DMR2) region of the *Igf2* gene known to augment transcription through methylation are significantly hypermethylated in insulinomas of *Men1* β -cell mutant mice at 6 and 10 months of age, even before IGF2 overexpression can be detected. Thus, our data indicate the involvement of both genetic and epigenetic mechanisms in early tumorigenesis of β -cells related to *MEN1* inactivation.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome transmitted with an autosomal dominant trait. The disease is characterized by the occurrence of multiple endocrine tumours of the parathyroids, pancreas and anterior pituitary (Thakker 1995, Online Mendelian Inheritance in Man no. 131100). Different laboratories have detected germline mutations of the *MEN1* gene in about 70–90% of familial MEN1

patients (Agarwal *et al.* 1997, Bassett *et al.* 1998, Giraud *et al.* 1998). Somatic mutations were also found in a substantial proportion of several sporadic endocrine tumours, especially in insulinoma, gastrinoma and parathyroid adenoma (Zhuang *et al.* 1997, Farnebo *et al.* 1998). The mutations revealed in the above analyses showed a typical ‘loss of function’ profile, establishing no genotype–phenotype correlation (Wautot *et al.* 2002). The loss of heterozygosity frequently observed in MEN1

tumours (Larsson *et al.* 1988) supports the hypothesis that the *MEN1* gene acts as a tumour suppressor in affected cells.

To generate adequate tools for the study of the mechanisms involved in endocrine malignancy related to *MEN1* gene inactivation, we and others have generated *Men1* mutant mice using either conventional (Crabtree *et al.* 2001, Biondi *et al.* 2002, Bertolino *et al.* 2003b) or conditional gene targeting strategies (Bertolino *et al.* 2003c, Crabtree *et al.* 2003, Libutti *et al.* 2003, Biondi *et al.* 2004). Heterozygous *Men1* mutant mice start to develop the major endocrine tumours seen in *MEN1* patients at around 12 months of age, whereas homozygous *Men1* mutant embryos die at E11.5–E13.5 with multiple developmental defects (Crabtree *et al.* 2001, Bertolino *et al.* 2003a). In parallel, pancreatic β -cell- and parathyroid-specific *Men1* gene disruption results in the development of insulinoma (Bertolino *et al.* 2003c, Biondi *et al.* 2004, Crabtree *et al.* 2003) and parathyroid adenoma respectively (Libutti *et al.* 2003). We have noticed that the insulinoma started to appear in β -cell-specific *Men1* mutant mice at around 6 months of age (early stage insulinoma). At 10 months, all the β -cell-specific *Men1* mutant mice developed insulinomas with adenocarcinoma features (late stage insulinoma). Furthermore, mouse *Men1* insulinomas in this model appeared not only earlier than those found in heterozygous *Men1* mutant mice, but also relatively synchronized (Bertolino *et al.* 2003c), making such mice a suitable model for dissecting the genetic events that occur during the tumour initiation and progression. Using this model, we have previously demonstrated the existence of a long interval period between the appearance of *menin*-inactivated cells and the development of insulinoma. This prompted us to propose the hypothesis that the tumorigenesis of β -cells triggered by the disruption of the *Men1* gene needs the participation of other factors (Bertolino *et al.* 2003c).

To identify these factors, we carried gene expression profiling of the insulinomas developed in β -cell-specific *Men1* mutant mice at 6 and 10 months of age, corresponding respectively to the insulinomas at early and late stages (Bertolino *et al.* 2003c). Our results reveal a substantial number of genes, whose expression is either up- or down-regulated in tumours. More importantly, our data provide evidence that the gene expression profile of the insulin-like growth factor (IGF) and cell cycle pathways is particularly deregulated. Further analysis reveals that the over-expression of IGF2 is accompanied by the hyper-methylation of the intragenic DMR2 region containing elements that increase the level of transcription

through methylation. Our work highlights the early involvement of both genetic and epigenetic mechanisms in tumorigenesis of β -cells related to *MEN1* inactivation.

Materials and methods

Men1^{F/F}-RipCre⁺ pancreatic β -cell-specific *Men1* mutant mice

As described previously, pancreatic β -cell-specific *Men1* mutant mice were generated by crossing homozygous mice carrying the floxed allele (*Men1^{F/F}*) with RipCre transgenic mice expressing the Cre recombinase under the control of the rat insulin promoter, termed *Men1^{F/F}*-RipCre⁺ mice (Bertolino *et al.* 2003c). All animal experiments were conducted in accordance with accepted standards of humane animal care and were approved by IARC's Animal Care and Use Committee.

Isolation and culture of mouse pancreatic islets

Pancreatic islets were isolated from mice of 2–10 months age according to the protocol previously described (Bertolino *et al.* 2003c). Briefly, 2 ml of 1 mg/ml collagenase (SERVA, Heidelberg, Germany) in Hank's buffered saline solution was injected into the pancreas through the bile duct. Pancreases were then removed and incubated for 20 min at 37 °C and dissociated by mechanical pipetting. Islets were hand-picked from dark field dishes under a dissecting microscopy.

RNA extraction

Total RNA from the islets were prepared using the RNeasy mini-kit (Qiagen) according to the protocol provided by the manufacturer, including the additional step of DNase treatment. Total RNA yield was measured using OD₂₆₀ (optical density), and the quality of isolated total RNA was evaluated with the agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) for microarray analysis or on a 1% ethidium bromide stained-gel for quantitative PCR.

Microarray analysis

RNA amplification

Total RNA, 2 μ g, was amplified and labelled by a round of *in vitro* transcription using the message amp aRNA kit (Ambion, TX, USA) following the manufacturer's protocol. Before amplification, all tubes were spiked with synthetic mRNA at different concentrations in order to verify the quality of the

amplification. aRNA yield was measured with an u.v. spectrophotometer and quality verified on nanochips with the Agilent 2100 bioanalyzer (Agilent Technologies).

Array hybridization and processing

Biotin labelled aRNA, 10 µg was fragmented with 5 µl fragmentation buffer in a final volume of 20 µl. Fragmented aRNA were added to the hybridization solution (GE Healthcare Europe GmbH, Freiburg, Germany) in a final volume of 260 µl and injected into the CodeLink Uniset Mouse I Bioarrays containing 10 000 mouse genes probes (GE Healthcare). Arrays were hybridized overnight at 37 °C at 300 rpm. The slides were washed in a stringent buffer containing 0.1 M Tris-HCl pH 7.6, 0.15 M NaCl and 0.05% Tween 20 at 46 °C for 1 h followed by a streptavidin-Cy5 (GE Healthcare) detection step. Each slide was incubated in 3.4 ml streptavidin-Cy5 solution for 30 min. After this incubation, the slides were washed four times in 240 ml TNT buffer. For the final washes, the slides were rinsed twice in 240 ml water containing 0.2% triton. Slides were finally dried by centrifugation at 600 rpm.

Slides were scanned using a Genepix 4000B scanner (Axon Instruments, Union City, CA, USA) and Genepix software, with the laser set at 635 nm, laser power at 100% and photomultiplier tube voltage at 60%. The scanned image files were analyzed using CodeLink expression software, version 4.0 (GE Healthcare), which produces both raw and normalized hybridization signals for each spot on the arrays.

Microarray data analysis

The overall raw hybridization signal intensity of arrays was normalized using CodeLink expression software version 4.0 (GE Healthcare) by setting the raw hybridization signal on each array as ratio to the median of the array (median intensity is 1 after normalization) for better cross-array hybridization. This study used normalized signal intensities. Probes containing missing data were eliminated from the list. The threshold of detection was calculated using the normalized signal intensity of the 100 negative controls represented in the array. Spots with signal intensity below the threshold were termed 'absent'.

Quality of processing was evaluated by generating scatter plot representations of positive signal distribution. Signal intensities were then transformed by logarithm (based 2). A differential expression of at least 4 (minimal fold change) was retained in order to

generate the final list of deregulated genes, whereas the average fold change is used when describing individually the expression level of genes of interest. Statistical comparison and filtering were achieved by using the Genespring software 7.0 (Agilent Technologies).

Quantitative PCR

Reverse transcription (RT)

Total RNA, 1 or 2 µg was reverse transcribed with 0.5 µg of oligo(dT) using M-MLV reverse transcriptase (Invitrogen). The absence of contaminating genomic DNA of the RT reactions was controlled by qPCR performed directly on total RNA.

Quantitative PCR

First-strand cDNA was used for real-time PCR with the Light cycler instrument and FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Primers were designed using Primer3 software (primer3_www.cgi v 0.2 – Whitehead Institute/MIT, USA) and their sequences shown in Supplementary Table 1, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. Serially diluted cDNA samples were used to calculate the efficiency of each PCR with RealQuant software (Roche). After an initial Taq activation at 95 °C for 10 min, Light Cycler PCR was performed using 45 cycles with the following cycling conditions: 95 °C for 15 s, 62 °C for 5 s and 72 °C for 16 s. Directly after the PCR, the machine performed a melting curve analysis by slowly (0.1 °C/s) increasing the temperature from 68 to 95 °C.

Quantitative PCR analysis

The level of hypoxanthine guanine phosphoribosyl transferase (HPRT) transcript was used as an internal standard to control the amplification variations due to the differences in the starting mRNA concentrations. The mean of the control samples has been set as 1, to normalize the results.

Statistical analysis

Mann–Whitney *U*-test was used to compare means between two groups with $P < 0.05$ considered as significant.

Histological and immunohistochemical analysis

Pancreases were collected from mice, fixed in 4% buffered formalin for 24 h and embedded in paraffin.

Serial sections (3 µm) were stained with haematoxylin and eosin (H&E) or immunostained as described previously (Bertolino *et al.* 2003c) using the following antibodies: anti-cyclin A recognising mouse cyclin A2 (H-432, 1:200, Santa-Cruz Biotechnology, Santa-Cruz, CA, USA), anti-cyclin D2 (M-20, 1:500, Santa-Cruz Biotechnology), anti-IGF-II (1:1000, Monoclonal; Upstate, Charlottesville, VA, USA) and anti-Menin (1:200, Polyclonal; Santa-Cruz Biotechnology).

Pyrosequencing analysis

DNA was extracted from five different tissues (endocrine pancreas, exocrine pancreas, adrenal, pituitary and tail). DNA concentration was determined using the Quant-iT dsDNA broad range assay kit (Invitrogen) and normalized to a concentration of 50 ng/µl. One microgram DNA was bisulphite converted as described in detail elsewhere (Dupont *et al.* 2004). Quantitative DNA methylation analysis of the bisulphite-treated DNA was performed by pyrosequencing or, in the case of several sequencing primers, by serial pyrosequencing (Tost *et al.* 2006). Regions of interest were amplified using 30 ng bisulphite-treated mouse genomic DNA and 5 pmol forward and reverse primers, one of them being biotinylated. Sequences of oligonucleotides for PCR amplification and pyrosequencing are shown in Supplementary Table 2, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. The reaction was performed in 1 × HotStar Taq buffer supplemented with 1.6 mM MgCl₂, 200 mM dNTPs and 2.0 U HotStar Taq polymerase (Qiagen) in a 25 µl volume. The PCR program consisted of a 15 min denaturing step at 95 °C, followed by 50 cycles of 30 s at 95 °C, 30 s at the respective annealing temperature (Supplementary Table 2, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>) and 20 s at 72 °C, with a final extension of 5 min at 72 °C. Amplification products were purified and rendered single-stranded on a Pyrosequencing workstation (Pyrosequencing AB, Uppsala, Sweden) according to the manufacturer's instructions. Quantitative DNA methylation analysis was carried out on a PSQ 96MA system with the PyroGold SQA Reagent Kit (Pyrosequencing) and the results were analyzed using the Q-CpG software (Pyrosequencing). Mann–Whitney *U*-test was used to compare quantitative methylation values between the two groups.

Results

Gene expression profile of mouse insulinomas developed in *Men1* β-cell-specific mutant mice

To determine the alteration of gene expression in mouse *Men1* insulinomas at different stages during tumour initiation and progression, mRNAs were isolated from hand-picked pancreatic islets of two *Men1*^{F/F} mice, two *Men1* β-cell-specific mutant mice at 6 months and two at 10 months of age. These samples were subject to cDNA array analysis in two independent experiments using CodeLink UniSet Mouse Bioarray (Amersham) that contains 10 000 mouse genes, the data from both series of analysis being taken into account for individual gene expression evaluation. Our data revealed a total of 56 overexpressed and 194 repressed genes with more than fourfold minimum fold change in insulinomas from the mutant mice at 10 months compared with the controls (Table 1, Supplementary Table 3, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). The majority of these genes are involved in signal transduction, transcription, metabolism of protein, cell cycle, metabolism of carbohydrate and cell differentiation (Fig. 1), with the genes from the first group (signal transduction) consisting of 18.4% of all deregulated genes at 10 months, compared with its distribution (14.2%) within entire expressed genes (Supplementary Table 4, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Among the pancreas-specific transcriptional factors, *Pax6* and *Foxa2* were up-regulated (1.6- and 2.1-fold respectively) in insulinomas from the mutant mice at 10 months of age when compared with the controls, and *Foxa1* was down-regulated (2.9-fold), while *Pax4* and *NeuroD1* had no change. *Pdx1* and *Hlxb9* are not present on our microarray. We also noticed that the expression of several genes involved in carbohydrate and lipid metabolism were deregulated (Fig. 1, Supplementary Table 4, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>), indicating that the molecular basis of glucose and lipid homeostasis in β-cells is functionally disturbed. Moreover, the altered expression was found with many factors involved in cell cycle control, likely due to the abnormal proliferation of tumour cells. Interestingly, there were only very few genes that altered their expression more than four folds in insulinomas from *Men1* β-cell-specific mutant mice at 6 months of age, with three out of ten such genes belonging to the transcription group. We noticed that several genes that are down-regulated are physiologically expressed mainly in islet cell lineages other than β-cells, such as somatostatin, pancreatic polypeptide and

Table 1 Partial list of genes with differential expression in 6- and 10-month mouse *Men1* insulinomas compared with controls*Genes differentially expressed between controls and 6 months insulinomas*

Up-regulated genes

NM_007495	Astn1	Astrotactin	NM_011854	Oasl2	2'-5' Oligoadenylate synthetase-like 2
NM_019446	Barhl1	BarH-like 1	NM_009130	Scg3	Secretogranin III
NM_009945	Cox7a3	Cytochrome c oxidase, subunit VIIa 3			

Down-regulated genes

NM_018866	Scyb13	Small inducible cytokine subfamily B, member 13	NM_007598	Cap1	Adenylyl cyclase-associated CAP protein homolog 1
NM_019812	Sirt1	Sirtuin 1	NM_019395	Fbp1	Fructose biphosphatase 1

Genes differentially expressed between controls and 6 months insulinomas

Up-regulated genes

NM_007765	Crmp1	Collapsin response mediator protein 1	NM_019548	Maged3	Melanoma antigen, family D, 3
NM_053071	Cox6c	Cytochrome c oxidase, subunit Vic	NM_008970	Pthlh	Parathyroid hormone-like peptide
NM_009945	Cox7a3	Cytochrome c oxidase, subunit VIIa 3	NM_019642	Rpn2	Ribophorin II (Rpn2)
NM_009920	Cnil	Cornichon (<i>Drosophila</i>)-like	NM_009065	Rin	RAS-like protein expressed in neuron
NM_011854	Oasl2	2'-5' Oligoadenylate synthetase-like 2	NM_008858	Prkcm	Protein kinase C, mu
NM_007495	Astn1	Astrotactin	NM_009130	Scg3	Secretogranin III
NM_019446	Barhl1	BarH-like 1	NM_007906	Eef1a2	Eukaryotic translation elongation factor 1 alpha 2
NM_007388	Acp5	Acid phosphatase 5, tartrate resistant	NM_023612	Esm1	Endothelial cell-specific molecule 1
NM_016707	Bcl11a	B-cell CLL/lymphoma 11A	NM_016917	Slc39a1	Solute carrier family 39

Down-regulated genes

NM_008124	Gjb1	Gap junction membrane channel protein beta 1	NM_022032	Perp	p53 Apoptosis effector related to Pmp22
NM_008101	Gcgr	Glucagon receptor	NM_013639	Prph1	Peripherin
NM_008006	Fgf2	Fibroblast growth factor 2	NM_009521	Wnt3	Wingless-related MMTV integration site 3
NM_007482	Arg1	Arginase 1, liver	D83144	Six3	mRNA for Six 3a, complete cds
NM_010253	Gal	Galanin	NM_010020	Slc6a3	Solute carrier family 6, member 3
NM_008116	Ggtp	Gamma-glutamyl transpeptidase	NM_009215	Smst	Somatostatin
NM_010000	Cyp2b9	Cytochrome P450, 2b9, phenobarbital inducible, type a	NM_008456	Klk5	Kallikrein 5
NM_007442	Alx4	Aristaless 4	NM_008918	Ppy	Pancreatic polypeptide
NM_007557	Bmp7	Bone morphogenetic protein 7	NM_011168	Pripf	Prolactin-like protein F
NM_010810	Mmp7	Matrix metalloproteinase 7	NM_009042	Reg1	Regenerating islet-derived 1
NM_007559	Bmp8b	Bone morphogenetic protein 8b	NM_030553	Olfr7b	Olfactory receptor 7b
NM_011260	Reg3g	Regenerating islet-derived 3 gamma	NM_011259	Reg3a	Regenerating islet-derived 3 alpha

Fold change is ≥ 4 .

vasointestinal peptide. The down-regulation of these genes is thus most likely due to the reduced proportion of these cells in *Men1* insulinomas.

Clustering analysis highlighting the deregulation of the Hox, IGF and cell cycle pathways

To gain further insights into the altered gene expression in mouse *Men1* insulinomas, we performed

clustering analysis of the data. Previous studies have reported that menin is required for the regulation of several members of the *Hox* gene family through its interaction with MLL1/2 protein complex, including *Hoxa9* (Yokoyama *et al.* 2004), *Hoxc6* and *Hoxc8* (Hughes *et al.* 2004). Indeed, our analysis showed that among 19 *Hox* genes with detectable expression levels, 5 were down-regulated (Fig. 2A), including *Hoxa3* (4.3-fold), *Hoxb3* (3.7-fold), *Hoxb5* (2.2-fold), *Hoxb13*

Figure 1 Functional distribution of differentially expressed known genes in control islets versus 6- or 10-month mouse *Men1* insulinomas. Gene functions were established based on the annotation provided by the Gene Ontology database. Hatched bars represent genes that are down-regulated compared with controls; solid bars represent genes that are over-expressed compared with controls; The genes having minimal fold change ≥ 4 are included.

(3-fold) and *Hoxc13* (3-fold) as compared with the control. *Hoxa9* and *Hoxc8* were not expressed in either insulinoma or control samples, whereas *Hoxc6* was unchanged in insulinomas compared with the controls. Our data indicate that menin inactivation in islets affects the expression of other members of the family that are previously not known to be regulated by MLL1/2-menin complexes.

The IGF pathway is known to play an important role in β -cell proliferation and differentiation, as well as in glucose homeostasis (Vasavada et al. 2006). At 10 months of age, the expression of many members of this pathway is altered in mouse *Men1* insulinoma (Fig. 2B). *Igf1* and *Igfbp6* are down-regulated more than fivefold, whereas *Igfbp3* and *Igf2* are up-regulated 2.1- and 4.5-fold respectively. Several proteins involved in cell cycle control have previously been reported to be important for the control of the islet cell proliferation, especially *cyclin D1* (Cozar-Castellano et al. 2004), *CDK4* (Rane et al. 1999, Marzo et al. 2004) and *p27* (Uchida et al. 2005). Our data showed that *cyclin D1* was not detectable in both insulinomas and control samples, *p16*, *p18* and *p19* expressed similarly in insulinomas and controls, whereas *p27* is not included in our microarray. However, we found that many other genes in this group are subject to altered gene expression (Fig. 2C), particularly the

increased expression of *cyclin A2* (2.9-fold), *cyclin B2* (2.9-fold), *cyclin D2* (1.5-fold) and *p21* (1.6-fold) in insulinomas at 10 months of age.

Validation of gene expression alteration by quantitative RT-PCR

In order to confirm the microarray analysis, we analyzed the mRNA expression level of 13 selected genes by real-time quantitative RT-PCR. To this end, we used a total of 6 mRNA samples from *Men1*^{F/F} mice with four at 3–6 months and two at 10 months, 7 and 6 from *Men1* β -cell-specific mutant mice at 6 and 10 months respectively, including those used for the microarray. The altered expression of 11 genes out of 13 tested was confirmed by quantitative RT-PCR (see Table 2), as was the reduction of *Men1* expression in late tumour samples. Moreover, we noticed that *Fgf9* and *Mapk8ip1* expression was up-regulated only in three out of six insulinomas at 10 months (data not shown). Their variable expression in mouse *Men1* insulinomas may explain why the overexpression of these two genes revealed by microarray analysis has not been validated by quantitative RT-PCR. The validation of the majority of the candidate genes revealed by microarray indicates the quality and the reliability of the microarray analysis performed. We also noticed that, in general, the fold changes of gene expression revealed by quantitative RT-PCR correlate well with that obtained with microarray analysis, except for *PthrP*. In the latter case, although its overexpression was detected by both methods, the fold change obtained by quantitative RT-PCR is much less than that obtained by microarray analysis. In contrast, an alteration in *PthrP* expression was found, though weakly, by quantitative RT-PCR in *Men1* insulinomas at 6 months of age, but was not detected by microarray analysis. The discrepancy may be explained by its very low expression level in the controls (under the background level in microarray analysis), leading to fold change bias and the sample variation in different mice with quantitative PCR analysis at 10 months (data not shown). The data obtained for the genes involved in the IGF pathway, including *Igf2*, *Igfbp3*, and *Igfbp6*, and in cell cycle control pathways, including *cyclin A2*, *cyclin B2*, *cyclin D2* were shown in Fig. 3. All these genes showed a significantly altered expression by quantitative PCR analysis, except *Igfbp3*, which nevertheless presented a clear overexpression in three out of six samples tested. Importantly, by quantitative PCR analysis, the increased expression of *cyclin A2*, *cyclin*

Figure 2 Specific gene cluster analysis of controls islets, 6- and 10-month insulinomas. Each row represents a single gene and each column represents a single control or tumour sample. The results are shown as relative expression level compared to the mean of the controls. Red and green indicate expression levels above and below the mean respectively. A, *Hox* gene cluster. B, IGF pathway cluster. C, Cell cycle cluster.

B2 and *cyclin D2* was detected in *Men1* β -cell-specific mutant mice at 6 months, indicating that their expression change is the early molecular events during the tumour development.

Shortly after our quantitative RT-PCR validation work, Lee *et al.* has reported effects of the *Rip-Cre* transgene on glucose metabolism (Lee *et al.* 2006). To make sure that the gene alteration observed above was not due to the influence of the *Rip-Cre*⁺ transgene, we have checked the expression of several validated genes in the parental Cre-expressing mice strain, including *cyclin A2*, *cyclin B2* and *IGF2*. The results showed that the expression of all these three genes in the islets from *Rip-Cre*⁺ mice was similar to those from *Men1*^{F/F} mice, but differed significantly from mouse *Men1* insulinomas in a similar way to what found when comparing the islets from *Men1*^{F/F} mice with those from mouse *Men1* insulinomas (Supplementary Fig. 1, which may be viewed online at <http://erc.endocrinology-journals.org/supplemental/>).

IGF2, cyclin A2 and cyclin D2 overexpression detected by immunohistochemical staining in *Men1* insulinomas

We further attempted to investigate the local protein expression of IGF2, cyclin A2 and cyclin D2 in mouse *Men1* insulinoma. By immunohistochemistry (IHC) analysis, we found that IGF2 was overexpressed in insulinomas from all the tested mice (seven out of seven mice) at around 10 months of age. It is noted that, from the same *Men1* mutant mouse, IGF2 overexpression was found only in some of the insulinomas (Fig. 4F) where one insulinoma presented an overt IGF2 expression, but the neighbouring one did not. Also, IGF2 overexpression appeared either diffused (Fig. 4F) or focused (Fig. 4I) in insulinomas, suggesting that it may have both autocrine and paracrine effects. Immunostaining of both cyclin A2 and cyclin D2 in islets from *Men1*^{F/F} mice showed that their expression present a typical cell cycle-dependant pattern, with 20–30% and 35–40% of cells showing

Table 2 Comparison of the qPCR results with the microarray results

Access No	Name	6 months			10 months		
		Micro-array Fold change	q PCR		Micro-array Fold change	q PCR	
			Fold change	P value		Fold change	P value
Up-regulation							
AK004995	Bmp1	—	—		↑3.3	↑3.2	0.0022
NM_010514	IGF2	—	—		↑4.5	↑4	0.0022
NM_013518	Fgf9	—	↑1.2	0.0350	↑3.6	—	
NM_011162	Mapk8ip	—	↑1.3	0.0221	↑2.7	—	
NM_008827	Pgf	—	↑1.3	0.0221	↑3.1	↑2.3	0.0173
NM_008970	PthrP	—	↑1.8	0.0140	↑16.6	↑2.4	0.0152
NM_009828	Ccna2	—	↑2.7	0.0047	↑2.9	↑2.9	0.0087
NM_016707	Bcl11a	↑2.5	↑2.7	0.0012	↑4.5	↑3	0.0043
NM_007765	Crmp1	↑2.8	↑4.4	0.0012	↑9.9	↑7.8	0.0022
NM_009829	Ccnd2	—	↑1.3	0.0051	—	—	
NM_007630	Ccnb2	↑1.5	↑3.7	0.0025	↑2.9	—	
Down-regulation							
NM_019395	Fbp1	↓8.4	—		↓4.6	↓2.6	0.0411
NM_008344	IGFbp6	↓1.8	—		↓5.1	↓2.8	0.0411
AF024513	Men1	ND	↓1.8	0.0023	ND	↓12.6	0.0022

The difference was considered significant when *P*-value was ≤0.05.

strong positive staining for cyclin A2 and cyclin D2 respectively (Fig. 4J, M). On the contrary, the proportion of strong positive cells was markedly increased in mouse *Men1* insulinomas to more than 70% on average for cyclin A2 (Fig. 4K and L), and more than 80% on average for cyclin D2 (Fig. 4N and O). The increase of cyclin A2 and cyclin D2-positive cells was observed in most of the mutant mice tested regardless of the age (two out of two, and two out of three for insulinomas at 6 and 10 months of age for cyclin A2, and two out of two for insulinomas both at 6 and 10 months of age for cyclin D2). Taken together, the detection of IGF2, cyclin A2 and cyclin D2 overexpression by IHC analysis in mouse *Men1* insulinomas indicates that their deregulated expression may be among the crucial events participating in the tumorigenesis of β -cells triggered by *menin* inactivation.

Core DMR2 of the *Igf2* gene is hyper-methylated in mouse *Men1* insulinomas

It is known that the expression of IGF2 is regulated by both genetic and epigenetic mechanisms. We have thus checked the methylation status of regulatory regions of the *Igf2* gene. To this end, DNA methylation patterns on 81 CpGs in the DMR0, DMR1 and DMR2 as well as in other CpG-rich regions situated in exons and introns of the gene were analysed by pyrosequencing. Pyrosequencing has become an increasingly

popular technology for the analysis of DNA methylation patterns as it combines important features such as ease-of-use, robustness and quantitative accuracy with high resolution. It is a sequencing-by-synthesis method converting the amount of pyrophosphate released upon nucleotide incorporation into a luminometric signal of proportional intensity. It thereby permits the accurate quantification of multiple CpGs in a sequence of up to 150 nucleotides without the need for laborious sequencing and cloning. Although all regions exhibited some, but not significant, variability of the methylation level at consecutive CpGs (Fig. 5), only DNA extracted from the insulinomas at 6 and 10 months of age showed a significant hyper-methylation at five CpGs of the DMR2 when compared with control mice (*P* values between $P=4.6\times10^{-5}$ and $P=0.0002$ for each of the CpGs analysed individually, and $P<1\times10^{-10}$ when all CpGs taken into account). The insulinomas ($N=15$) had a mean methylation independent of age of $65.6\pm7.0\%$ compared with $51.3\pm3.1\%$ in the control (*Men1*^{F/F}) mice ($N=6$). No statistically significant difference in the methylation levels was found between conditional knockout mice at 6 ($N=8$) and 10 months ($N=7$) of age ($66.9\pm8.7\%$ at 10 months vs $64.6\pm6.0\%$ at 6 months of age). This hyper-methylation was confined only to the endocrine pancreas, and was not observed in any of the other analysed organs (tail, adrenal, pituitary and exocrine pancreas) in the same mice. Methylation levels were

Figure 3 Quantitative PCR analysis of candidate genes of the microarray. mRNA levels of *Igf2*, *Igfbp6*, *Igfbp3* (A), *cyclin A2*, *cyclin B2* and *cyclin D2* (B) were determined from a minimum of five samples of control islets, 6- (6m) and 10-month (10m) insulinomas by quantitative PCR. Hprt was used as an internal standard. Bars represent the median. * $P < 0.05$. † $P < 0.01$.

unchanged in all the five analyzed tissues in the control mice. No difference was found between male and female mice. To ensure that the increase in methylation was not due to the influence of the *Rip-Cre*⁺ mice, the DMR2 was also analyzed in the parental Cre expressing strain. The obtained methylation profile was identical to that of the control mice ($51.0 \pm 3.1\%$ *Rip-Cre*⁺ ($N=3$) vs $51.3 \pm 3.1\%$ *Men1*^{F/F} ($N=6$). Four of the hyper-methylated CpGs are situated in the core DMR2 and coincide with the region previously shown to increase transcription of the growth factor when methylated (Murrell *et al.* 2001). IGF2 is also regulated by an imprinting control region situated upstream of the *H19* gene containing four binding sites for the insulator CTCF protein (CCCTC-binding factor). We therefore additionally analysed the DNA methylation patterns at two of the CTCF sites (CTCF sites 1 and 4), and no significant difference was found between mouse *Men1* insulinomas and control islets (data not shown).

We have also analyzed the methylation patterns at promoters of the genes involved in cell cycle or proliferation controls and known to be regulated by epigenetic mechanisms, including *p27*, *p18* and *PTHRP* genes. However, we failed to reveal any significant change in mouse *Men1* insulinomas (data not shown).

Discussion

In the current study, we have characterized the gene expression profile of insulinomas developed in *Men1* β -cell mutant mice at early and late stages using microarray analysis. The results showed that the expression of a substantial number of genes is deregulated in tumorigenesis of β -cells, beginning from the mouse *Men1* insulinomas at 6 months of age. More importantly, with the combined analyses, our data allowed us to highlight the early involvement of both genetic and epigenetic events taking part in the tumorigenesis of the pancreatic β -cells triggered by menin inactivation.

Among the previously known islet-related growth-promoting factors, *PthrP* was found to be significantly overexpressed in mouse *Men1* insulinomas from both mice at 6 and 10 months of age. *Fgf9*, previously found upregulated in human MEN1 islet tumours (Dilley *et al.* 2005), was also overexpressed, though slightly, in mouse *Men1* insulinomas at 6 months. However, the expression of *Myc*, known to be involved in β -cell tumorigenesis (for review see Pelengaris & Khan 2001), was not altered. Suppression of apoptosis is considered to be an important event in both human and mouse β -cell tumorigenesis (Pelengaris & Khan 2001), and it has been reported previously that MEN1 islet

Figure 4 IGF2, cyclin A2 and cyclin D2 proteins in mouse *Men1* insulinomas. A, D, G: H&E staining. B, E, H: Immunostaining of menin. C, F, I: Immunostaining of IGF2. J-L: Immunostaining of cyclin A2. M-O: Immunostaining of D2. A-C, J, M: pancreas from *Men1^{F/F}-RipCre⁻* mice at 12 months. D-I, L, N: insulinomas from *Men1^{F/F}-RipCre⁺* mice at 12 months. K, O: insulinomas from *Men1^{F/F}-RipCre⁺* mice at 6 months. Inserts show an amplified view of a part of the islet. The scale bars are 50 μ m.

tumours have an altered expression of several apoptosis genes (Dilley et al. 2005). Interestingly, in the current study, the expression of both genes having pro- or anti-apoptotic functions was found to be altered. This may result from the integrated effects of

abnormal cell proliferation and cell death induced by certain growth factors. We have also checked the expression of proteins known to interact with menin in our tumour samples, such as JunD, Smad3 and NF κ B factors, and none of these factors showed altered

Figure 5 Methylation profile of the different analysed regions in the *Igf2* gene. A. The methylation profile obtained for the exocrine pancreas derived from the *Men1*^{F/F}-RipCre⁺ mice is identical to the profile obtained from the adrenal, pituitary and the tail. The profile shown for the endocrine pancreas of the *Men1*^{F/F} mice is representative for all tissues of the *Men1*^{F/F} control mice as well as the RipCre⁺ mice. Significant methylation differences are confined to 5 CpGs in the DMR2, of which four consecutive ones are in the core DMR2. B. Box-plot diagrams for the methylation differences at these four consecutive CpGs are shown. **P* < 0.001, ns, no statistical significant difference.

transcriptional expression. We have observed an increased expression of secretogranin III starting from 6 months, consistent with a previous report showing the strong expression of protein/insulin secretion pathway in human insulinoma tissue (Wang *et al.* 2004). Finally, among the genes with altered expression in 6 months insulinomas, we noticed that astrotactin, a membrane protein involved in cell migration, was upregulated both in the early and the late stages of tumour progression, suggesting that the alteration of cell migration may be an important feature of mouse *Men1* insulinomas.

Our data highlight that the gene expression alteration of cell cycle control genes may play an important role in tumorigenesis of mouse β -cells related to menin inactivation. Interestingly, whereas cyclin D1, whose overexpression has been reported to occur frequently in human pancreatic endocrine

tumours (Chung *et al.* 2000), did not show significant changes in expression in this model, both microarray and quantitative RT-PCR analyses demonstrated that cyclin A2, B2 and D2 were significantly up-regulated in insulinomas at the early stage. Moreover, the overexpression of cyclin A2 and D2 has been further confirmed by immunostaining analysis, with the results showing that the proportion of the cells expressing these two factors increased in tumour cells. Recently, cyclin D2 has been identified as the major D-type cyclin expressed in β -cells and essential for β -cells expansion (Kushner *et al.* 2005). However, the functional role of cyclin A2 and B2 in islet cells is so far poorly documented. Our data provide the first genetic evidence indicating that regulating the expression of these cyclins may be an important mechanism for menin to control β -cell proliferation. As little is known so far concerning the regulation of these cyclins in islet cells by menin, it will be interesting for future studies to investigate and study the mechanism of interplay between menin and these cyclins in islet cells. Taken together, the overexpression of cyclin A2, B2 and D2 may suggest that these gene alterations lead to the major early disturbance of cell cycle control in mouse *Men1* insulinomas.

Our data highlighted the altered expression of the IGF pathway, including the down-regulation of IGF1 and IGFbp6, and the up-regulation of IGF2 and IGFbp3. The down-regulation of *Igf1* expression detected in our mouse *Men1* insulinoma model seems to be consistent with the observation that *Igf1* pancreas-specific disruption led to β -cell hypertrophy and increased cell survival (Lu *et al.* 2004). IGFbp3 overexpression has been previously reported in pancreatic endocrine neoplasm and in other cancers, particularly in metastatic pancreatic endocrine neoplasm (Maitra *et al.* 2003, Hansel *et al.* 2004). Indeed, we noticed that half of the tested insulinoma samples from *Men1* mutant mice at 10 months (three out six) presented overt IGFbp3 overexpression, whereas no significant alteration could be observed with samples from mice at 6 months, suggesting that IGFbp3 overexpression is a non-systematic and late event in this model. As for IGF2, its overexpression has been detected by both quantitative RT-PCR and immunostaining analyses in all the mice tested in the current study. IGF2 overexpression has been well-documented in abnormal β -cells proliferation (Vasavada *et al.* 2006). Devedjian *et al.* (2000) have demonstrated that pancreases from transgenic mice overexpressing IGF2 showed an increase in β -cell mass, disordered organization of islets, hyperinsulinemia, mild hyperglycaemia, and altered glucose and insulin tolerance.

Furthermore, its expression has been detected in human islet tumours, especially in insulinomas (Hoog et al. 2001), and its overexpression was also observed in a mouse Rip-Tag insulinoma model (Christofori et al. 1994). Therefore, the up-regulation of IGF2 expression seen in this study could be considered as one of the major factors taking part in the tumorigenesis of β -cells, triggered by menin inactivation.

We have adopted pyrosequencing for evaluating the methylation levels in the regulatory regions of the *Igf2* gene, which permits the direct and accurate quantification of the methylation levels at multiple CpGs in a sequence of up to 120 base pairs without the need for time-consuming and laborious cloning and sequencing. Very interestingly, by analysing the methylation status of all the three DMRs of the *Igf2* gene, we have found that five CpG sites of the core DMR2 were all hyper-methylated in islets lesions. Methylation of the same four of five CpG sites has been previously reported to augment *Igf2* expression by increasing the level of transcription initiation (Murrell et al. 2001). To our knowledge, this is the first time where the hyper-methylation of this regulatory element is documented in tumour tissues. Furthermore, the hyper-methylation of these elements was found in islet lesions from *Men1* β -cell mutant mice at 6 months of age, even before IGF2 overexpression is found at the transcriptional level, suggesting that abnormal methylation of these silencing sites is an early event. Our work thus provided an important clue to the molecular mechanism leading to IGF2 overexpression observed in mouse *Men1* insulinoma. It will be interesting to investigate whether there is a causal relation between IGF2 overexpression and the observed hyper-methylation at DMR2 region, and whether the same epigenetic alteration happens in human insulinoma or other cancers. Considering menin's role in histone methylation (Hughes et al. 2004, Yokoyama et al. 2004, Milne et al. 2005) and acetylation (Kim et al. 2003) and the interaction between these process and DNA methylation, it would not be surprising that menin may also play a role, directly or indirectly, in DNA methylation.

In this study, we took advantage of the synchronized and homogenous tumour development features of insulinomas derived in *Men1* β -cell mutant mice to identify the factors taking part in tumour initiation and progression triggered by menin inactivation. Our data highlight several early genetic and epigenetic events that are of importance in the development of mouse *Men1* insulinomas, including the overexpression of IGF2 and cyclin A2, B2 and D2. Furthermore, the overexpression of IGF2 is most likely mediated by a

hyper-methylation mechanism that is associated with the absence of menin. Further studies of mouse *Men1* insulinomas may shed light onto the physiological control of β -cell proliferation and also onto the underlying mechanisms of both metabolic diseases and tumours affecting pancreatic β -cell.

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